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(54) Title: METHOD OF DIAGNOSING PULMONARY HYPERTENSION

(57) Abstract: This invention relates generally to a method of identifying an individual having an increased susceptibility to devel-  
oping Familial Primary Pulmonary Hypertension (FPPH), as well as to a method for diagnosing an individual suffering from FPPH.  
The invention also relates to a method of identifying an individual having an increased susceptibility to developing (non-familial)  
Primary Pulmonary Hypertension (PPH), as well as to a method for diagnosing an individual suffering from PPH.

## METHOD OF DIAGNOSING PULMONARY HYPERTENSION

### FIELD OF THE INVENTION

This invention relates generally to a method of identifying an individual having an increased susceptibility to developing Familial Primary Pulmonary Hypertension (FPPH), as well as to a method for diagnosing an individual suffering from FPPH. The invention also relates to a method of identifying an individual having an increased susceptibility to developing non-familial, or sporadic, Primary Pulmonary Hypertension (PPH), as well as to a method for diagnosing an individual suffering from sporadic PPH. The invention also relates to a method of identifying an agent capable of altering the symptoms of PPH in an individual suffering from familial or sporadic PPH, comprising contacting a test agent with Bone Morphogenic Protein Receptor II (BMPR-II) and determining whether the test agent alters BMPR-II activity, wherein an alteration in BMPR-II activity in the presence of the test agent as compared with BMPR-II activity in the absence of the test agent indicates that the test agent is capable of altering the symptoms of PPH in an individual suffering from familial or sporadic PPH.

### BACKGROUND OF THE INVENTION

Primary pulmonary hypertension (PPH) is characterized by sustained elevation of pulmonary artery pressure (greater than 25 mmHg at rest and greater than 30 mmHg during exercise) and with no identifiable cause, such as recurrent thromboembolism, chronic hypoxic lung disease or left-sided cardiac disease. PPH is twice as common in females than males and symptoms develop typically in the 3<sup>rd</sup> and 4<sup>th</sup> decades of life, although the disease may occur at any age. Despite advances in therapy, mortality in PPH remains high with mean survival from onset of disease only 2.5 year.

At least 6 % of individuals diagnosed with PPH have a known family history of the disorder. The disease can be classified as being either familial (more than one affected relative has been identified in at least 6% of cases (familial PPH; MIM 178600) (ref. 3)) or sporadic. Familial PPH (FPPH) segregates as an autosomal dominant disorder, with markedly reduced penetrance.

There is a need to identify the genetic basis for this devastating disease in order to better diagnose and treat patients suffering from PPH.



### BRIEF SUMMARY OF THE INVENTION

The invention relates to a method of identifying a subject having an increased susceptibility for developing pulmonary hypertension, comprising detecting a mutant Bone Morphogenic Protein Receptor II (BMPR-II) polypeptide or a mutated Bone Morphogenic Protein Receptor 2 (BMPR2) nucleic acid in the subject, thereby  
5 identifying a subject having an increased susceptibility for developing pulmonary hypertension. Wild-type BMPR2 nucleotide sequence is SEQ ID NO:1. Wild-type BMPR-II amino acid sequence is SEQ ID NO:2.

In one aspect, the mutated BMPR2 nucleic acid or mutant BMPR-II polypeptide  
10 has a sequence associated with pulmonary hypertension.

In another aspect, the mutated BMPR2 nucleic acid comprises a missense mutation.

In yet another aspect, the mutated BMPR2 nucleic acid comprises a nonsense mutation.

15 In another aspect, the mutated BMPR2 nucleic acid comprises a deletion mutation.

In another aspect, the mutated BMPR2 nucleic acid comprises an insertion mutation.

In another aspect, the mutated BMPR2 nucleic acid comprises a truncation  
20 mutation. Preferably, the mutated BMPR2 nucleic acid is truncated at a nucleotide position of the sequence set forth in SEQ ID NO:1 which is 3' to nucleotide position 2695.

In another aspect, the subject having an increased susceptibility for developing pulmonary hypertension is identified by detecting a BMPR2 nucleic acid having a  
25 sequence associated with pulmonary hypertension.

In a preferred aspect, the pulmonary hypertension is primary pulmonary hypertension. In another aspect, the pulmonary hypertension is secondary pulmonary hypertension.

In various preferred embodiments, the mutated BMPR2 nucleic acid can include  
30 a missense mutation or a nonsense mutation.

In another aspect, the invention features a method of identifying a mutant BMPR-II polypeptide or a mutated BMPR2 nucleic acid, including detecting, in a patient with PPH, a BMPR-II polypeptide that is not present in normal subjects or a BMPR2 nucleic acid that is not present in normal subjects, thereby identifying a mutant  
5 BMPR-II polypeptide or a mutated BMPR2 nucleic acid.

In another aspect, the invention features a method of increasing BMPR-II biological activity.

In another aspect, the invention features a method of decreasing BMPR-II biological activity.

10 In another aspect, the invention features a method of identifying a compound that modulates the biological activity of a BMPR-II polypeptide, including: a) contacting a sample including a BMPR-II polypeptide or a BMPR2 nucleic acid with the compound; and b) measuring BMPR-II biological activity in the sample, whereby an increase or decrease in BMPR-II biological activity, compared to BMPR-II  
15 biological activity in an identical sample not contacted with the compound, identifies a compound that modulates the biological activity of the BMPR-II polypeptide.

In various embodiments of this aspect of the invention, BMPR-II biological activity is increased or decreased; the BMPR-II polypeptide is a wild-type BMPR-II polypeptide or the BMPR2 nucleic acid is a wild-type BMPR2 nucleic acid; the  
20 BMPR-II polypeptide is a polymorphic variant of a BMPR-II polypeptide or the BMPR2 nucleic acid is a polymorphic variant of a BMPR2 nucleic acid; or the BMPR-II polypeptide is a mutant BMPR-II polypeptide or the BMPR2 nucleic acid is a mutated BMPR2 nucleic acid.

In another aspect, the invention features a non-human mammal having a  
25 deleted, mutated, or polymorphic variant BMPR2 gene. In various aspects of the twelfth aspect of the invention, the non-human mammal is a mouse; and/or the non-human mammal is homozygous for the deleted, mutated, or polymorphic variant BMPR2 gene.

Additional advantages of the invention will be set forth in part in the description  
30 which follows, and in part will be obvious from the description, or may be learned by practice of the invention. The advantages of the invention will be realized and attained

by means of the elements and combinations particularly pointed out in the appended claims. It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed.

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### BRIEF DESCRIPTION OF THE DRAWING

Figures 1A-1C are diagrams showing the physical map of the PPH1 interval at 2q33. Figure 1A shows the position of microsatellite markers. The markers are, from left to right, D2S115, D2S348, D2S2392, D2S2396, D2S1367, D2S116, D2S309, D2S2309, D2S2214, D2S2217, D2S346, D2S2289, 19E07, D2S307, CTLA4, D2S72, 10 D2S105, D2S2189, D2S1384. Figure 1B shows the physical map contig (BAC/PAC) of the region surrounding BMPR2 including other genes analysed in the examples (12). Figure 1C shows the BMPR2 genomic structure, determined by analysis of available sequence data for BAC clone RP11-345N12 as well as sequence analysis of additional BAC clones identified by library screening as shown (not to scale).

15

Figure 2A is a diagram of the structure of BMPR2 cDNA. The location of the exons are indicated by the nucleotide start position in the cDNA. The cysteine residues within the extracellular domain are each denoted by \*. The filled in box represents the transmembrane domain and the stippled area identifies the region encoding the receptor kinase domain.

20

Figure 2B is a diagram of BLAST homology results showing protein similarity of human BMPR-II with receptors in other species and human TGF- $\beta$  receptor type II (TGFBR-II). Amino acid positions are shown together with the codon substitutions of conserved amino acids (boxed).

25

Figure 3 is a diagram of a basic function of BMPR-II. Two BMP type I receptors (BMPRIA and BMPRIB) and a single BMP type II receptor have been identified in mammals as serine/threonine kinase receptors. Following ligand binding to BMPR-II, this receptor forms a heteromeric complex with a type I receptor, resulting in activation of the type I receptor kinase domain which initiates phosphorylation of cytoplasmic signalling proteins, termed Smads, responsible for signal transduction.

## DETAILED DESCRIPTION OF THE INVENTION

The invention relates to the surprising discovery that FPPH is caused by mutations in the gene encoding a TGF- $\beta$  type II receptor, BMPR-II. The invention provides a method of identifying a subject having an increased susceptibility for developing pulmonary hypertension, comprising detecting a mutant Bone Morphogenic Protein Receptor II (BMPR-II) polypeptide or a mutated BMPR2 nucleic acid in the subject, thereby identifying a subject having an increased susceptibility for developing pulmonary hypertension. BMPR2 refers to the gene (or other nucleic acid) encoding a BMPR-II polypeptide. BMPR-II refers to the polypeptide encoded by a BMPR2 gene. Both of these terms are used herein as general identifiers. Thus, for example, a BMPR2 gene or nucleic acid refers to any gene or nucleic acid identified with or derived from a wild-type BMPR2 gene. For example, a mutant BMPR2 gene is a form of BMPR2 gene.

In a preferred embodiment, the pulmonary hypertension is primary pulmonary hypertension. In another embodiment, the pulmonary hypertension is secondary pulmonary hypertension.

As used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, "a molecule" can mean a single molecule or more than one molecule.

By "about" is meant  $\pm 10\%$  of a recited value.

By "BMPR-II biological activity" is meant any physiological function attributable to a BMPR-II polypeptide molecule, including signal transduction. BMPR-II biological activity, as referred to herein, is relative to that of the normal BMPR-II polypeptide molecule. It may be desirable to increase or decrease BMPR-II biological activity.

Mechanisms by which a compound may increase BMPR-II biological activity include, but are not limited to, mimicry of endogenous BMPR-II polypeptide activity; stimulation of the activity of a less active or inactive version (for example, a mutant) of the BMPR-II polypeptide; or increasing the amount of BMPR-II polypeptide in a cell

(for example, by stimulating BMPR2 transcription and/or translation or by inhibiting BMPR2 mRNA or polypeptide degradation).

5 BMPR-II biological activity in a sample, such as a cell, tissue, or animal, may be indirectly measured by measuring the relative amount of BMPR2 mRNA (for example, by reverse transcription-polymerase chain reaction (RT-PCR) amplification, ribonuclease protection assay or Northern hybridization); the level of BMPR-II polypeptide (for example, by ELISA or Western blotting); or the activity of a reporter gene under the transcriptional regulation of a BMPR2 transcriptional regulatory region (by reporter gene assay, for example, employing beta-galactosidase, chloramphenicol  
10 acetyltransferase (CAT), luciferase, or green fluorescent protein, as is well known in the art). For example, a compound that increases the amount of wild-type BMPR-II polypeptide (or any other version of the polypeptide that maintains at least some activity) in a cell is a compound that increases biological activity of BMPR-II.

By "BMPR-II polypeptide" is meant a polypeptide that has, or is related to, the  
15 amino acid sequence of SEQ ID NO:2. A BMPR-II polypeptide contains an amino acid sequence that bears at least 80% sequence identity, preferably at least 85% sequence identity, more preferably at least 90% sequence identity, and most preferably at least 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO:2.

20 By "wild-type BMPR-II polypeptide" is meant a BMPR-II polypeptide that has the amino acid sequence of SEQ ID NO:2.

By "wild-type BMPR2 nucleic acid" is meant a nucleic acid that encodes a wild-type BMPR-II polypeptide. An example of a wild-type BMPR2 nucleic acid is SEQ ID NO:1. Other wild-type BMPR2 nucleic acids include those containing introns,  
25 such as genomic BMPR2 nucleic acid.

By "polymorphic variant of a BMPR-II polypeptide" is meant a BMPR-II polypeptide containing an amino acid change, relative to wild-type, that does not result in an increase susceptibility to PPH. Such polymorphic amino acid variations in BMPR-II are seen in both PPH patients and in normal individuals.

30 By "mutant BMPR-II polypeptide" is meant a BMPR-II polypeptide having an amino acid sequence that differs from the sequence of a wild-type BMPR-II



polypeptide. One example of a wild-type BMPR-II polypeptide is a polypeptide having the amino acid sequence set forth in SEQ ID NO:2.

By "mutated BMPR2 nucleic acid" is meant a nucleic acid having a nucleotide sequence that differs from the sequence of the wild-type BMPR2 nucleic acid. One  
5 example of a wild-type BMPR2 nucleic acid is a nucleic acid having the nucleotide sequence set forth in SEQ ID NO:1. A "mutated nucleic acid" is also a nucleic acid that encodes a BMPR-II polypeptide having an amino acid sequence that differs from the sequence of a wild-type BMPR2 polypeptide. One example of a wild-type BMPR-II polypeptide is a polypeptide having the amino acid sequence set forth in SEQ ID  
10 NO:2. A mutated nucleic acid also includes a nucleic acid having a mutation (relative to the wild-type nucleic acid) in noncoding sequences, such as 5' or 3' sequences or intronic sequences.

By "increased susceptibility for developing pulmonary hypertension" is meant a subject who has a greater than normal chance of developing pulmonary hypertension,  
15 compared to the general population. Such subjects include, for example, a subject that harbors a mutation in a BMPR2 gene such that biological activity of BMPR-II is altered.

By "test compound" is meant a molecule, be it naturally occurring or artificially derived, that is surveyed for its ability to modulate BMPR-II activity. Test compounds  
20 may include, for example, peptides, polypeptides, synthesized organic molecules, naturally occurring organic molecules, nucleic acid molecules, and components thereof.

By "sample" is meant an animal; a tissue or organ from an animal; a cell (either within a subject, taken directly from a subject, or a cell maintained in culture or from a cultured cell line); a cell lysate (or lysate fraction) or cell extract; or a solution  
25 containing one or more molecules derived from a cell or cellular material (e.g. a polypeptide or nucleic acid), which is assayed as described herein. A sample may also be any body fluid or excretion (for example, but not limited to, blood, urine, stool, saliva, tears, bile) that contains cells or cell components.

By "modulate" is meant to alter, by increase or decrease.

30 By "normal subject" is meant an individual who does not have an increased susceptibility for developing pulmonary hypertension.

By an "effective amount" of a compound as provided herein is meant a sufficient amount of the compound to provide the desired effect. The exact amount required will vary from subject to subject, depending on the species, age, and general condition of the subject, the severity of disease (or underlying genetic defect) that is being treated, the particular compound used, its mode of administration, and the like. Thus, it is not possible to specify an exact "effective amount." However, an appropriate "effective amount" may be determined by one of ordinary skill in the art using only routine experimentation.

By "isolated polypeptide" or "purified polypeptide" is meant a polypeptide (or a fragment thereof) that is substantially free from the materials with which the polypeptide is normally associated in nature. The polypeptides of the invention, or fragments thereof, can be obtained, for example, by extraction from a natural source (for example, a mammalian cell), by expression of a recombinant nucleic acid encoding the polypeptide (for example, in a cell or in a cell-free translation system), or by chemically synthesizing the polypeptide. In addition, polypeptide fragments may be obtained by any of these methods, or by cleaving full length polypeptides.

By "isolated nucleic acid" or "purified nucleic acid" is meant DNA that is free of the genes that, in the naturally-occurring genome of the organism from which the DNA of the invention is derived, flank the gene. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector, such as an autonomously replicating plasmid or virus; or incorporated into the genomic DNA of a prokaryote or eukaryote (e.g., a transgene); or which exists as a separate molecule (for example, a cDNA or a genomic or cDNA fragment produced by PCR, restriction endonuclease digestion, or chemical or *in vitro* synthesis). It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence. The term "isolated nucleic acid" also refers to RNA, e.g., an mRNA molecule that is encoded by an isolated DNA molecule, or that is chemically synthesized, or that is separated or substantially free from at least some cellular components, for example, other types of RNA molecules or polypeptide molecules.

By a "transgene" is meant a nucleic acid sequence that is inserted by artifice into a cell and becomes a part of the genome of that cell and its progeny. Such a

transgene may be (but is not necessarily) partly or entirely heterologous (for example, derived from a different species) to the cell.

By "transgenic animal" an animal comprising a transgene as described above. Transgenic animals are made by techniques that are well known in the art.

5 By "knockout mutation" is meant an alteration in the nucleic acid sequence that reduces the biological activity of the polypeptide normally encoded therefrom by at least 80% relative to the unmutated gene. The mutation may, without limitation, be an insertion, deletion, frameshift, or missense mutation. A "knockout animal," for example, a knockout mouse, is an animal containing a knockout mutation. The  
10 knockout animal may be heterozygous or homozygous for the knockout mutation. Such knockout animals are generated by techniques that are well known in the art. A preferred form of knockout mutation is one where the biological activity of the BMPR-II polypeptide is not completely eliminated.

By "treat" is meant to administer a compound or molecule of the invention to a  
15 subject, such as a human or other mammal (for example, an animal model), that has an increased susceptibility for developing pulmonary hypertension, or that has pulmonary hypertension, in order to prevent or delay a worsening of the effects of the disease or condition, or to partially or fully reverse the effects of the disease.

By "prevent" is meant to minimize the chance that a subject who has an  
20 increased susceptibility for developing pulmonary hypertension will develop pulmonary hypertension.

By "specifically binds" is meant that an antibody recognizes and physically interacts with its cognate antigen (for example, a BMPR-II polypeptide) and does not significantly recognize and interact with other antigens; such an antibody may be a  
25 polyclonal antibody or a monoclonal antibody, which are generated by techniques that are well known in the art.

By "probe," "primer," or oligonucleotide is meant a single-stranded DNA or RNA molecule of defined sequence that can base-pair to a second DNA or RNA molecule that contains a complementary sequence (the "target"). The stability of the  
30 resulting hybrid depends upon the extent of the base-pairing that occurs. The extent of base-pairing is affected by parameters such as the degree of complementarity between

the probe and target molecules and the degree of stringency of the hybridization conditions. The degree of hybridization stringency is affected by parameters such as temperature, salt concentration, and the concentration of organic molecules such as formamide, and is determined by methods known to one skilled in the art. Probes or  
5 primers specific for BMPR2 nucleic acids (for example, genes and/or mRNAs) have at least 80%-90% sequence complementarity, preferably at least 91%-95% sequence complementarity, more preferably at least 96%-99% sequence complementarity, and most preferably 100% sequence complementarity to the region of the BMPR2 nucleic acid to which they hybridize. Probes, primers, and oligonucleotides may be detectably-  
10 labeled, either radioactively, or non-radioactively, by methods well-known to those skilled in the art. Probes, primers, and oligonucleotides are used for methods involving nucleic acid hybridization, such as: nucleic acid sequencing, reverse transcription and/or nucleic acid amplification by the polymerase chain reaction, single stranded conformational polymorphism (SSCP) analysis, restriction fragment polymorphism  
15 (RFLP) analysis, Southern hybridization, Northern hybridization, *in situ* hybridization, electrophoretic mobility shift assay (EMSA).

By "specifically hybridizes" is meant that a probe, primer, or oligonucleotide recognizes and physically interacts (that is, base-pairs) with a substantially complementary nucleic acid (for example, a BMPR2 nucleic acid) under high  
20 stringency conditions, and does not substantially base pair with other nucleic acids.

By "high stringency conditions" is meant conditions that allow hybridization comparable with that resulting from the use of a DNA probe of at least 40 nucleotides in length, in a buffer containing 0.5 M NaHPO<sub>4</sub>, pH 7.2, 7% SDS, 1 mM EDTA, and 1% BSA (Fraction V), at a temperature of 65°C, or a buffer containing 48% formamide,  
25 4.8X SSC, 0.2 M Tris-Cl, pH 7.6, 1X Denhardt's solution, 10% dextran sulfate, and 0.1% SDS, at a temperature of 42°C. Other conditions for high stringency hybridization, such as for PCR, Northern, Southern, or *in situ* hybridization, DNA sequencing, etc., are well-known by those skilled in the art of molecular biology. See, for example, F. Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley &  
30 Sons, New York, NY, 1998.

By "familial mutation" or "inherited mutation" is meant a mutation in an individual that was inherited from a parent and that was present in somatic cells of the parent. By "sporadic mutation" or "spontaneous mutation" is meant a mutation in an individual that arose in the individual and was not present in a parent of the individual.

5 By "BMPR2 RNA function" is meant a function of the RNA other than the state of coding for an amino acid sequence. For example, BMPR2 RNA production, stability, processing (including splicing), transport, and the ability to be translated are BMPR2 RNA functions. By "altered BMPR2 RNA function" is meant an alteration of BMPR2 RNA function relative to the function of wild-type BMPR2 RNA.

10 As set forth herein, nucleotides are numbered according to the cDNA sequence for BMPR2 (SEQ ID NO:1), with the adenosine of the initiation codon assigned position 1. (Kawabata, M., Chytil, A. & Moses, H.L. Cloning of a novel type II serine/threonine kinase receptor through interaction with the type I transforming growth factor-beta receptor. *J. Biol. Chem.* **270**, 5625-5630 (1995); Liu, F., Ventura, F.,  
15 Doody, J. & Massagué, J. Human type II receptor for bone morphogenic proteins (BMPs): extension of the two-kinase receptor model to the BMPs. *Mol. Cell. Biol.* **15**, 3479-3486 (1995); Rosenzweig, B.L. *et al.* Cloning and characterization of a human type II receptor for bone morphogenetic proteins. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 7632-7636 (1995).

20 The nucleotide and amino acid sequence of BMPR2 are shown in SEQ ID NO:1 and SEQ ID NO:2, respectively, starting at nucleotide 1 and amino acid 1, respectively. However, the wild-type cDNA sequence for BMPR2 which is set forth in Genbank Accession No. NM\_001204, assigns the adenosine of the initiation codon to position 409. Therefore, nucleotide position 1 used herein corresponds to nucleotide number  
25 409 of the BMPR2 cDNA sequence set forth in Genbank Accession No. NM\_001204. Thus, where a mutation is noted as being at, for example, nucleotide residue 1454, this corresponds to nucleotide residue 1862 of the sequence set forth in Genbank Accession No. NM\_001204 (that is, 1454 + 408).

30 As used herein, a specific notation will be used to denote certain types of mutations. All notations referencing a nucleotide or amino acid residue will be understood to correspond to the residue number of the wild-type BMPR2 nucleic acid

sequence set forth at SEQ ID NO:1, or of the wild-type BMPR-II polypeptide sequence set forth at SEQ ID NO:2. Thus, for example, the notation "T 367 C" will be used to indicate that the nucleotide T at position 367 of the sequence set forth at SEQ ID NO:1 has been replaced with a C. Similarly, the notation "355 del A" will be used to indicate  
5 that the nucleotide A at position 355 has been deleted. Furthermore, the notation 2408 ins TG" will be used to indicate that the nucleotides T and G, in that order, have been inserted following the nucleotide at position 2408.

In the method of the invention, the mutant BMPR-II polypeptide or mutated BMPR2 nucleic acid identified is associated with pulmonary hypertension.

10 In one embodiment, the subject having an increased susceptibility for developing pulmonary hypertension is identified by detecting a mutated BMPR2 nucleic acid in the subject. The mutated BMPR2 nucleic acid may comprise a missense mutation, that is, a mutation that changes a codon specific for one amino acid to a codon specific for another amino acid. As is noted below in the Examples and in Tables  
15 1, 2, and 4, examples of mutated BMPR2 nucleic acids having a missense mutation which are associated with pulmonary hypertension include C 218 G, T 354 G, T 367 C, T 367 A, C 428 T, C 993 T, G 1042 A, T1258 C, A 1454 G, A 1535 C, T 1557 A, and C 2695 T.

In another embodiment, the BMPR2 nucleic acid having a sequence associated  
20 with pulmonary hypertension comprises a nucleic acid sequence having an insertion mutation, where one or more nucleotides are inserted into the wild-type sequence. The mutated BMPR2 nucleic acid may also comprise a deletion mutation, where one or more nucleotides are deleted from the wild-type sequence. Such a deletion or insertion mutation may, for example, result in a frameshift mutation, altering the reading frame.  
25 Frameshift mutations typically result in truncated (that is, prematurely terminated) BMPR-II polypeptide. As is noted below in the Examples and in Tables 1, 2, and 4, examples of BMPR2 nucleic acids having an insertion mutation which are associated with pulmonary hypertension include 504 ins T, 2292 ins A, and 2408 ins TG. Examples of BMPR2 nucleic acids having a deletion mutation which are associated  
30 with pulmonary hypertension include 355 del A, 689 del A, 958 del T, 1076 del C, 1191/1192 del TG, and 2579 del T.

The mutated BMPR2 nucleic acid may also comprise a nonsense mutation, that is, a mutation that changes a codon specific for an amino acid to a chain termination codon. Nonsense mutations result in truncated (that is, prematurely terminated) BMPR-II polypeptide. As is set forth below in the Examples and in Table 1, examples  
5 of BMPR2 nucleic acids having a nonsense mutation which are associated with pulmonary hypertension include C 218 G, C 428 T, C 993 T, and C 2695 T.

The mutated BMPR2 nucleic acid may also comprise a truncation mutation, that is, a mutated BMPR2 nucleic acid which encodes a truncated BMPR-II polypeptide. This may occur where, for example, the BMPR2 nucleic acid has a nonsense mutation.

10 In another embodiment, the mutated BMPR2 nucleic acid can be truncated at a nucleotide position of the sequence set forth in SEQ ID NO:1 which is 3' to nucleotide position 2695 of the sequence set forth at SEQ ID NO:1. As is set forth below in the Examples, it has been determined that a mutation at nucleotide 2695, which truncates the BMPR-II polypeptide at amino acid residue 899, is correlated to pulmonary  
15 hypertension.

In another embodiment, the mutated BMPR2 nucleic acid comprises a mutation at a nucleotide position of the sequence set forth in SEQ ID NO:1 selected from the group consisting of nucleotide 218, 354, 355, 367, 428, 504, 689, 958, 993, 1042, 1076, 1129, 1191, 1258, 1454, 1535, 1557, 1749, 2292, 2408, 2579, and 2695. The mutation  
20 can result in a change in a codon such that the mutated codon now encodes a different amino acid. The mutation can result in a polypeptide having a non-conservative substitution at the relevant amino acid residue. One of ordinary skill will readily understand the concept of a "non-conservative substitution." Substitutions such as a charged amino acid for an uncharged amino acid, or an uncharged amino acid for a  
25 charged amino acid, or any amino acid in place of a Cys, or visa versa, or any amino acid in place of a Pro, or visa versa, are well known in the art to alter the structure and often the function of a protein. The mutation can also result in reduction or elimination of BMPR2 mRNA production, incorrect or altered processing of BMPR2 RNA, increased BMPR2 RNA instability, or other effects on expression of BMPR2 prior to  
30 translation. For example, the mutation 1129 CG (Table 1) alters a splice junction and

results in incorrect splicing of BMPR2 RNA. The mutation C 1749 T, which does not alter the encoded amino acid, likely affects RNA production, processing, or function.

In the embodiment wherein the mutation in the mutated BMPR2 nucleic acid results in a non-conservative substitution in the amino acid sequence encoded by the nucleic acid, the mutation in the mutated BMPR2 nucleic acid can be selected from the group consisting of C 218 G, T 354 G, T 367 C, T 367 A, C 428 T, C 993 T, G 1042 A, T1258 C, A 1454 G, A 1535 C, T 1557 A, C 2695 T. The non-conservative substitution may comprise at least one substitution at an amino acid position of the sequence set forth in SEQ ID NO:2 selected from the group consisting of: a Trp residue at amino acid position 118, an Arg residue at amino acid position 123, a Ser residue at amino acid position 123, a Leu residue at amino acid position 143, an Ile residue at amino acid position 348, an Arg residue at amino acid position 420, an Ala residue at amino acid position 485, a Gln residue at amino acid position Gln, and a Lys residue at amino acid position 519.

In yet another embodiment, the BMPR2 nucleic acid having a sequence associated with pulmonary hypertension encodes a mutant BMPR-II polypeptide.

For example, the mutant BMPR-II polypeptide having a sequence associated with pulmonary hypertension can comprise at least one mutation at an amino acid position of the sequence set forth in SEQ ID NO:2. Preferably, the BMPR-II polypeptide comprises at least one mutation at an amino acid position of the sequence set forth in SEQ ID NO:2 selected from the group consisting of 73, 118, 123, 143, 332, 348, 420, 485, 512, 519, and 899.

For example, the BMPR-II polypeptide acid having a sequence associated with pulmonary hypertension may comprise at least one mutation at an amino acid position of the sequence set forth in SEQ ID NO:2 selected from the group consisting of: a Trp residue at amino acid position 118, an Arg residue at amino acid position 123, a Ser residue at amino acid position 123, a Leu residue at amino acid position 143, an Ile residue at amino acid position 348, an Arg residue at amino acid position 420, an Ala residue at amino acid position 485, a Gln residue at amino acid position Gln, and a Lys residue at amino acid position 519.



In another embodiment, the BMPR-II polypeptide having a sequence associated with pulmonary hypertension terminates prematurely. In a preferred embodiment, the BMPR-II polypeptide having a sequence associated with pulmonary hypertension terminates at an amino acid position of the sequence set forth in SEQ ID NO:2 which is at or N-terminal to amino acid position 899, including amino acid positions 73, 332, and 899.

In another embodiment, the BMPR-II polypeptide having a sequence associated with pulmonary hypertension has a non-conservative amino acid substitution of at least one amino acid residue of a BMPR-II having the amino acid sequence set forth in SEQ ID NO:2.

In one embodiment, the non-conservative amino acid substitution comprises a non-conservative amino acid substitution of any of the following: at least one Cys residue of a BMPR-II having the amino acid sequence set forth in SEQ ID NO:2. In another embodiment, the non-conservative amino acid substitution comprises a non-conservative amino acid substitution of at least one Pro residue of a BMPR-II having the amino acid sequence set forth in SEQ ID NO:2. In another embodiment, the non-conservative amino acid substitution comprises a non-conservative amino acid substitution of at least one Lys residue of a BMPR-II having the amino acid sequence set forth in SEQ ID NO:2. In another embodiment, the non-conservative amino acid substitution comprises a non-conservative amino acid substitution of at least one Arg residue of a BMPR-II having the amino acid sequence set forth in SEQ ID NO:2. In another embodiment, the non-conservative amino acid substitution comprises a non-conservative amino acid substitution of at least one Asp residue of a BMPR-II having the amino acid sequence set forth in SEQ ID NO:2. In another embodiment, the non-conservative amino acid substitution comprises a non-conservative amino acid substitution of at least one Glu residue of a BMPR-II having the amino acid sequence set forth in SEQ ID NO:2.

The mutated BMPR2 nucleic acid and mutant BMPR-II polypeptide that is detected can be from any cause. For example, mutated BMPR2 nucleic acid can be the result of a familial mutation or a sporadic mutation.

### Kits

The disclosed method is preferably carried out using a kit designed or adapted to detect one or more mutant BMPR-II polypeptides and/or one or more mutated BMPR2 nucleic acids. An example would be a kit for detecting a variety of mutated BMPR2 nucleic acids. Many such kits, and methods for using them are known.

### Nucleic Acid Delivery

BMPR-II biological activity can be stimulated (or correct activity provided) in a subject by administering to the subject a nucleic acid encoding BMPR-II, using any method known for nucleic acid delivery into the cells of a subject. The BMPR2 nucleic acid is taken up by the cells of the subject and directs expression of the encoded BMPR-II in those cells that have taken up the nucleic acid. The BMPR2 nucleic acids of the present invention can be in the form of naked DNA or RNA, or the nucleic acids can be within a vector for delivering the nucleic acids to the cells. The vector can be a commercially available preparation, such as an adenovirus vector (Quantum Biotechnologies, Inc. (Laval, Quebec, Canada). Delivery of the nucleic acid or vector to cells can be via a variety of mechanisms. As one example, delivery can be via a liposome, using commercially available liposome preparations such as LIPOFECTIN, LIPOFECTAMINE (GIBCO-BRL, Inc., Gaithersburg, MD), SUPERFECT (Qiagen, Inc. Hilden, Germany) and TRANSFECTAM (Promega Biotec, Inc., Madison, WI), as well as other liposomes developed according to procedures standard in the art. In addition, the nucleic acid or vector of this invention can be delivered *in vivo* by electroporation, the technology for which is available from Genetronics, Inc. (San Diego, CA) as well as by means of a SONOPORATION machine (ImaRx Pharmaceutical Corp., Tucson, AZ).

As one example, vector delivery can be via a viral system, such as a retroviral vector system which can package a recombinant retroviral genome (see e.g., Pastan et al., *Proc. Natl. Acad. Sci. U.S.A.* 85:4486, 1988; Miller et al., *Mol. Cell. Biol.* 6:2895, 1986). The recombinant retrovirus can then be used to infect and thereby deliver to the infected cells a nucleic acid that encodes a BMPR-II polypeptide. The exact method of introducing the altered nucleic acid into mammalian cells is, of course, not limited to the use of retroviral vectors. Other techniques are widely available for this procedure

including the use of adenoviral vectors (Mitani et al., *Hum. Gene Ther.* 5:941-948, 1994), adeno-associated viral (AAV) vectors (Goodman et al., *Blood* 84:1492-1500, 1994), lentiviral vectors (Naidini et al., *Science* 272:263-267, 1996), pseudotyped retroviral vectors (Agrawal et al., *Exper. Hematol.* 24:738-747, 1996). Physical  
5 transduction techniques can also be used, such as liposome delivery and receptor-mediated and other endocytosis mechanisms (see, for example, Schwartzenberger et al., *Blood* 87:472-478, 1996). The present invention can be used in conjunction with any of these or other commonly used gene transfer methods.

In a particular example, to deliver a BMPR2 nucleic acid to the cells of a human  
10 subject in an adenovirus vector, the dosage can range from about  $10^7$  to  $10^9$  plaque forming unit (pfu) per injection but can be as high as  $10^{12}$  pfu per injection (Crystal, *Hum. Gene Ther.* 8:985-1001, 1997; Alvarez and Curiel, *Hum. Gene Ther.* 8:597-613, 1997). Ideally, a subject will receive a single injection. If additional injections are necessary, they can be repeated at six month intervals for an indefinite period and/or  
15 until the efficacy of the treatment has been established.

Parenteral administration of the nucleic acid or vector of the present invention, if used, is generally characterized by injection. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution of suspension in liquid prior to injection, or as emulsions. A more recently  
20 revised approach for parenteral administration involves use of a slow release or sustained release system such that a constant dosage is maintained. See, e.g., U.S. Patent No. 3,610,795. For additional discussion of suitable formulations and various routes of administration of therapeutic compounds, see, e.g., *Remington: The Science and Practice of Pharmacy* (19th ed.) ed. A.R. Gennaro, Mack Publishing Company,  
25 Easton, PA 1995.

### Animal Models

Compounds identified as modulating BMPR2 or BMPR-II expression or proposed to affect PPH may be subsequently screened in any available animal model system, including, but not limited to, mice, rats, pigs, rabbits, and chickens (Smith, JD,  
30 *Lab. Anim. Sci.* 48:573-579, 1998; Narayanaswamy et al., *J. Vasc. Interv. Radiol.* 11:5-17, 2000; Poernama et al., *Atheroscler. Thromb.* 12:601-607, 1992; and Schreyer et al.,

*Atheroscler. Thromb.* 14:2053-2059, 1994). Test compounds are administered to these animals according to standard methods. Known animal models include monocrotalin injection, continuous air embolism, and fawn hooded rat. Some useful models are described by Johnson et al., Pulmonary veins and bronchial vessels undergo remodeling

5 in sustained pulmonary hypertension induced by continuous air embolization into sheep, *Experimental Lung Research*. 23(5):459-73 (1997); Perkett et al., Expression of transforming growth factor-beta mRNAs and proteins in pulmonary vascular remodeling in the sheep air embolization model of pulmonary hypertension, *American Journal of Respiratory Cell & Molecular Biology*. 11(1):16-24 (1994); Perkett et al.,

10 Insulin-like growth factor I and pulmonary hypertension induced by continuous air embolization in sheep, *American Journal of Respiratory Cell & Molecular Biology*. 6(1):82-7 (1992); Perkett et al., Sequence of structural changes and elastin peptide release during vascular remodelling in sheep with chronic pulmonary hypertension induced by air embolization, *American Journal of Pathology*. 139(6):1319-32 (1991);

15 Perkett et al., Continuous air embolization into sheep causes sustained pulmonary hypertension and increased pulmonary vasoreactivity, *American Journal of Pathology*. 132(3):444-54 (1988); Morio et al., Distal airspace enlargement in the fawn-hooded rat: influences of aging and alveolar wall destruction, *Respiration*. 68(1):78-86 (2001); Le

20 Cras et al., Early abnormalities of pulmonary vascular development in the Fawn-Hooded rat raised at Denver's altitude, *American Journal of Physiology - Lung Cellular & Molecular Physiology*. 279(2):L283-91 (2000); Le Cras et al., Abnormal lung growth and the development of pulmonary hypertension in the Fawn-Hooded rat, *American Journal of Physiology*. 277(4 Pt 1):L709-18 (1999); Gonzalez et al., The pulmonary hypertensive fawn-hooded rat has a normal serotonin transporter coding sequence,

25 *American Journal of Respiratory Cell & Molecular Biology*. 19(2):245-9 (1998); Gonzalez et al., Pulmonary hypertension, family and environment, *Journal of Human Hypertension*. 11(9):559-61 (1997); Provoost, Spontaneous glomerulosclerosis: insights from the fawn-hooded rat, *Kidney International - Supplement*. 45:S2-5 (1994); Sato et al., Factors influencing the idiopathic development of pulmonary hypertension in the

30 fawn hooded rat, *American Review of Respiratory Disease*. 145(4 Pt 1):793-7 (1992); Ashmore et al., Paradoxical constriction to platelets by arteries from rats with

pulmonary hypertension, American Journal of Physiology. 260(6 Pt 2):H1929-34 (1991); Nagaya et al., Gene transfer of human prostacyclin synthase ameliorates monocrotaline-induced pulmonary hypertension in rats, Circulation. 102(16):2005-10 (2000); Shubat et al., Pulmonary vascular responses induced by the pyrrolizidine alkaloid, monocrotaline, in rats, Toxicol. 25(9):995-1002 (1987); Gust and Schuster, Vascular remodeling in experimentally induced subacute canine pulmonary hypertension, Experimental Lung Research. 27(1):1-12 (2001); Ito et al., Alterations of endothelium and smooth muscle function in monocrotaline-induced pulmonary hypertensive arteries, American Journal of Physiology - Heart & Circulatory Physiology. 279(4):H1786-95 (2000); Tanabe et al., Experimental study on monocrotaline induced pulmonary hypertensive rats. (1) Effect of long-term injection of immunosuppressant, Tokai Journal of Experimental & Clinical Medicine. 6(1):41-8 (1981); and Kameji et al., Increase of collagen synthesis in pulmonary arteries of monocrotaline-treated rats, Experientia. 36(4):441-2 (1980).

Animal models that mimic PPH can be developed using conventional molecular biology methods. For example, a transgenic animal (for example, a mouse) that overexpresses BMPR2 can be generated by inserting a BMPR2-encoding nucleic acid under the transcriptional regulation of the appropriate tissue-specific promoter into the genome of the animal.

## Test Compounds

In general, novel drugs that modulate BMPR-II biological activity may be identified from large libraries of natural products or synthetic (or semi-synthetic) extracts or chemical libraries according to methods known in the art. Those skilled in the field of drug discovery and development will understand that the precise source of test extracts or compounds is not critical to the screening procedure(s) of the invention. Accordingly, virtually any number of chemical extracts or compounds can be screened using the exemplary methods described herein. Examples of such extracts or compounds include, but are not limited to, plant-, fungal-, prokaryotic- or animal-based extracts, fermentation broths, and synthetic compounds, as well as modification of existing compounds. Numerous methods are also available for generating random or directed synthesis (for example, semi-synthesis or total synthesis) of any number of

chemical compounds, including, but not limited to, saccharide-, lipid-, peptide-, and nucleic acid-based compounds. Synthetic compound libraries are commercially available, e.g., from Brandon Associates (Merrimack, NH) and Aldrich Chemical (Milwaukee, WI). Alternatively, libraries of natural compounds in the form of

5 bacterial, fungal, plant, and animal extracts are commercially available from a number of sources, including Biotics (Sussex, UK), Xenova (Slough, UK), Harbor Branch Oceanographics Institute (Ft. Pierce, FL), and PharmaMar, U.S.A. (Cambridge, MA). In addition, natural and synthetically produced libraries are generated, if desired, according to methods known in the art, for example, by standard extraction and

10 fractionation methods. Furthermore, if desired, any library or compound is readily modified using standard chemical, physical, or biochemical methods.

In addition, those skilled in the art of drug discovery and development readily understand that methods for dereplication (for example, taxonomic dereplication, biological dereplication, and chemical dereplication, or any combination thereof) or the

15 elimination of replicates or repeats of materials already known for their BMPR-II-modulatory activities should be employed whenever possible.

When a crude extract is found to modulate BMPR-II activity, further fractionation of the positive lead extract is necessary to isolate chemical constituents responsible for the observed effect. Thus, the goal of the extraction, fractionation, and

20 purification process is the careful characterization and identification of a chemical entity within the crude extract having an activity that mimics, stimulates, or antagonizes BMPR-II, depending upon the effect desired. The same assays described herein for the detection of activities in mixtures of compounds can be used to purify the active component and to test derivatives thereof. Methods of fractionation and purification of

25 such heterogenous extracts are known in the art. If desired, compounds shown to be useful agents for treatment are chemically modified according to methods known in the art. Compounds identified as being of therapeutic value can be subsequently analyzed using any animal models for PPH.

#### **Administration of compounds that modulate BMPR-II biological activity**

30 The compositions and methods described herein can be used therapeutically in combination with a pharmaceutically acceptable carrier. By "pharmaceutically

acceptable carrier" is meant a material that is not biologically or otherwise undesirable, that is, the material may be administered to an individual along with a polypeptide, nucleic acid, or other compound of the invention without causing any undesirable biological effects or interacting in a deleterious manner with any of the components of the pharmaceutical composition in which it is contained. Pharmaceutical carriers are  
5 well-known in the art. These most typically are standard carriers for administration of vaccines or pharmaceuticals to humans, including solutions such as sterile water, saline, and buffered solutions at physiological pH.

Molecules intended for pharmaceutical delivery may be formulated in a  
10 pharmaceutical composition. Pharmaceutical compositions may include carriers, thickeners, diluents, buffers, preservatives, surface active agents and the like in addition to the molecule of choice. Pharmaceutical compositions may also include one or more active ingredients such as antimicrobial agents, anti-inflammatory agents, anesthetics, and the like. Methods for making such formulations are well known in the art, and are  
15 described, for example, in: *Remington: The Science and Practice of Pharmacy* (19<sup>th</sup> ed.), ed. A.R. Gennaro, E.W. Martin Mack Publishing Co., Easton, PA, 1995.

The pharmaceutical compositions may be administered in a number of ways depending on whether local or systemic treatment is desired, and on the area to be treated. Administration may be topically (including ophthalmically, vaginally, rectally,  
20 intranasally), orally, by inhalation, or parenterally, for example by intravenous drip, subcutaneous, intraperitoneal or intramuscular injection. The compounds and compositions of the present invention can be administered intravenously, intraperitoneally, intramuscularly, subcutaneously, intracavity, or transdermally.

Preparations for parenteral administration include sterile aqueous or  
25 non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose,  
30 dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on

Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like.

Formulations for topical administration may include ointments, lotions, creams,  
5 gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable.

Compositions for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets, or tablets. Thickeners,  
10 flavorings, diluents, emulsifiers, dispersing aids or binders may be desirable. Formulations for parenteral administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives.

The compounds of the invention are administered in an effective amount, using standard approaches. Effective dosages and schedules for administering the  
15 compounds may be determined empirically, and making such determinations is routine to one of ordinary skill in the art. The skilled artisan will understand that the dosage will vary, depending upon, for example, the species of the subject the route of administration, the particular compound to be used, other drugs being administered, and the age, condition, sex and extent of the disease in the subject. The dosage can be  
20 adjusted by the individual physician in the event of any counterindications. A dose of a compound of the invention generally will range between about 1  $\mu\text{g/kg}$  of body weight and 1 g/kg of body weight. Examples of such dosage ranges are, e.g., about 1  $\mu\text{g}$ -100  $\mu\text{g/kg}$ , 100  $\mu\text{g/kg}$ -10 mg/kg, or 10 mg-1 g/kg, once a week, bi-weekly, daily, or two to four times daily. Compounds of the invention include BMPR-II polypeptides, BMPR2  
25 nucleic acids, and molecules that regulate expression and/or biological activity of endogenous wild-type, polymorphic, and/or mutant BMPR-II polypeptides and/or nucleic acids (for example, DNA or RNA molecules) encoding such BMPR-II polypeptides.

30 The following Examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how the compounds,



compositions, articles, devices and/or methods claimed herein are made and evaluated, and are intended to be purely exemplary of the invention and are not intended to limit the scope of what the inventors regard as their invention.

### Examples

#### 5 Example 1

This example describes identification of BMPR2 mutations associated with susceptibility to PPH. To enable positional cloning of the FPPH gene (*PPH1*) a published YAC map was verified and extended at 2q33 to anchor a BAC/PAC contig including genomic sequences available at GenBank (Hadano, S. *et al.* A yeast artificial  
10 chromosome-based physical map of the juvenile amyotrophic lateral sclerosis (ALS2) critical region on human chromosome 2q33-q34. *Genomics* 55, 106-112 (1999)). This contig covers the entire 5.8 Mb *PPH1* region defined by recently detected recombination events limited by the polymorphic STS markers *D2S115* and *D2S1384* (ref.12), and includes the nucleotide sequence encoding bone morphogenetic protein  
15 receptor type II (BMPR-II).

PCR primers were designed for amplification of patient genomic DNA after determination of the intron/exon boundaries of BMPR2. Sequence variants were identified from the panel of kindreds studied. Heterogeneous mutations, including frameshift, nonsense and missense mutations, were identified. These mutations were  
20 distributed across the gene.

Either restriction enzyme or sequence analysis of DNA from affected and unaffected first-degree relatives was used to show co-segregation of the mutations with the disease phenotype (including obligate gene carriers) in all but one of the pedigrees. 150 normal chromosomes derived from the same population as the affected families  
25 were screened together with a panel of 64 chromosomes from normal, but ethnically diverse, controls subjects. None of the mutations were detected in either panel.

### Materials and Methods

#### Patients

We ascertained families in which at least two members had the typical  
30 manifestations of PPH after exclusion of known associated disorders, as previously described (5). We collected venous blood samples and extracted genomic DNA

following informed consent. Obligate gene carriers are defined as those individuals who inherit and transmit the disease gene to an affected offspring but who themselves show no clinical manifestations of the disease.

#### **Determination of the genomic structure of BMPR2**

5 Available genomic sequence of BAC RP11-354N12  
([http://www.ncbi.nlm.nih.gov/ Genbank](http://www.ncbi.nlm.nih.gov/Genbank)) was compared to the published cDNA sequence (Genbank Z48923) and the intron/exon boundaries for the 3' portion of the gene (exons 8-13) determined (14). To determine the intron/exon boundaries for exons 1-7, additional BAC clones were isolated by PCR screening of a human genomic BAC  
10 library (CITB B&C, Research Genetics) using both an exon 1 and exon 3 STS designed from the BMPR2 cDNA. Direct sequence analysis of the BAC clones with primer predicted to be near intron/exon junctions, based on the mouse *Bmpr2* genomic structure, generated flanking intronic sequence for the remaining introns (18). Comparison between the human and published mouse genomic organization shows  
15 strong conservation of the intron/exon boundaries (18).

#### **Mutational analysis**

We screened the entire coding and intron/exon boundaries by direct sequencing of both forward and reverse strands on either an ABI 377 sequencer or an ABI 3700 DNA analyzer, using the Applied Biosystems DyeDeoxy or BigDye terminator kit and  
20 analysed the data using Sequence Analysis v3.2 or v3.6NT software (Perkin Elmer). The PCR primers for each exon were:

Exon1: 5'-AGCTAGGTCCTCTCATCAGC-3' (SEQ ID NO:3)

5'-CAGCCGCAGTGCTGACCAGC-3' (SEQ ID NO:4);

Exon2: 5' -GTCATTCGGATAAGACAAAG-3' (SEQ ID NO:5)

25 5'-TTTAACATACTCCCATGTCC-3' (SEQ ID NO:6);

Exon3: 5'-TAGCTTACACGTACTCTCAC-3' (SEQ ID NO:7)

5'-CCTGGCTTCAACCTTGAATG-3' (SEQ ID NO:8);

Exon4: 5'-GGGTACAGCCTTTCTAAAGG-3' (SEQ ID NO:9)

5'-GATACTATTGAGGCTGGGTG-3' (SEQ ID NO:10);

30 Exon5: 5'-GCTGCTAATCTTTCTGCAGC-3' (SEQ ID NO:11)

5'-GAATGAAGTCACTGTTCCAG-3' (SEQ ID NO:12);

- Exon6: 5'-CAGAGAGCTGTAGCATTCTG-3' (SEQ ID NO:13)  
 5'-AAGTGATCCACCTGCCTTAG-3' (SEQ ID NO:14);  
 Exon7: 5'-ACTCTTCATGTTAAAGTGAG-3' (SEQ ID NO:15)  
 5'-CTTTGAAGATATAATTAATAATTTCC-3' (SEQ ID NO:16);  
 5 Exon8: 5'-CACCTGGCCAGTAGATGTTT-3' (SEQ ID NO:17)  
 5'TGTTCAATAGTCCCTTTTATTCATTG-3' (SEQ ID NO:18);  
 Exon9: 5'-CTAATTTGCATCCTGCTGCT-3' (SEQ ID NO:19)  
 5'-TGTTCTTCAGAATATGCTACG TTCTC-3' (SEQ ID NO:20);  
 Exon10: 5'-TTGTGGCATTAGGCAACTCC-3' (SEQ ID NO:21)  
 10 5'-GCCTGAAGGGGATGAA AAA-3' (SEQ ID NO:22);  
 Exon11: 5'-CCACACCCCTTAGGGTCTTA-3' (SEQ ID NO:23)  
 5'-CACATGGTTTGACATGTAC TTTG-3' (SEQ ID NO:24);  
 Exon12A: 5'-CATCAGAGCTTTCCTTGAGGTT-3' (SEQ ID NO:25)  
 5'-CAGAGGTGTTAAATTT GGAG-3' (SEQ ID NO:26);  
 15 Exon12B: 5'-TCTACCTGCCACACCATTC-3' (SEQ ID NO:27)  
 5'-TGGAAACCAACAAGCTAGACC-3' (SEQ ID NO:28);  
 Exon12C: 5'-CCCCAAAAGACACACAGGAG-3' (SEQ ID NO:29)  
 5'-TGAATGGTGTGGCAGGTAGA-3' (SEQ ID NO:30);  
 Exon13: 5'-GCTGACAGGAGGATAAAGCA-3' (SEQ ID NO:31)  
 20 5'-CACCCCTCCTGAGACATTGGT-3' (SEQ ID NO:32).

#### **Restriction endonuclease digestion**

- We confirmed segregation of the mutations within families and excluded the presence of the mutations in controls, including a panel from the DNA Polymorphism Discovery Resource, Coriell Cell Repositories, by PCR amplification of the relevant  
 25 exon. This was followed by either mutation specific restriction fragment length polymorphism (RFLP) analysis or direct sequencing as previously described (23). Exon 12 (2579-2580delT) was PCR amplified using the following primers as a nested PCR reaction: 5' ACCCAATATGCCAATGGGAC-3' (SEQ ID NO:33), 5'-  
 TTCGCCACCTTCTAGTGGCT-3' (SEQ ID NO:34) followed by 5'-  
 30 CATGTGGTAAACTGAAAAGCTCA-3' (SEQ ID NO:35), 5'-  
 TTGAGACCACTTTGATACACACA-3' (SEQ ID NO:36). We digested an aliquot (10

μl) overnight at 37°C with the appropriate enzyme (10U; Gibco) and separated the fragments on a 4% agarose gel.

In keeping with the recognized reduced penetrance of FPPH, some individuals over 40 years of age exhibited the restriction fragments of a mutant but were not affected (individuals 6 and 10 in NL01). Nucleic acid mutations included A 1454 G (exon 11, *Ava*II, pedigree NL01), T 354 G (exon 3, *Bsp*1286I, pedigree US14), 2579-2580 delT (exon 12, *Ase*I, pedigree US55), C 2695 T (exon 12, *Hae*III, pedigree US33), 355 del A (exon 3, *Bsp*1286I, pedigree UK13), G 1042 A (exon 8, pedigree UK06), and C 218 G (exon 2, pedigree US35). Polypeptide mutations included D 485 G (pedigree NL01), C 118 W (pedigree US14), PTC + 10aa (pedigree US55), R 899 X (pedigree US33), PTC + 8aa (pedigree UK13), C 347 Y (pedigree UK06), and S 73 X (pedigree US35). The downstream amino acid position of the premature termination codon (PTC) is indicated by the designation "+ Xaa" where X is number of amino acids downstream of the codon that is mutated. Sequence analysis of both forward and reverse strands was performed for those PPH families in which the observed mutation did not create or destroy a restriction site: G 1042 A (exon 8, pedigree UK06), and C 218 G (exon 2, pedigree US35).

### Discussion

Members of the TGF-β superfamily transduce signals by binding to heteromeric complexes of type I and II receptors, activating serine/threonine kinases, leading to transcriptional regulation by phosphorylated Smads (Massagué, J. & Chen, Y-G. Controlling TGF-β signalling. *Genes Dev.* 14, 627-644 (2000)). In FPPH, mutations in the gene encoding BMPR-II lead to alterations in domains which have been identified in TGF-β type II receptors as being involved in ligand binding, kinase activity and heteromeric dimer formation (Wrana, J.L. *et al.* Two distinct transmembrane serine/threonine kinases from *Drosophila melanogaster* form an activin receptor complex. *Mol. Cell. Biol.* 14, 944-950 (1994); Carcamo, J., Zentella, A. & Massagué, J. Disruption of transforming growth factor beta signalling by a mutation that prevents transphosphorylation within the receptor complex. *Mol. Cell. Biol.* 15, 1573-1581 (1995); Gilboa, L. *et al.* Bone morphogenetic protein receptor complexes on the surface

of live cells: A new oligomerization mode for serine/ threonine kinase receptors. *Mol. Biol. Cell.* **11**, 1023-1035 (2000)).

### References

1. Rubin, L. ACCP consensus statement: primary pulmonary hypertension. *Chest*  
5 **104**, 236-250 (1993).
2. Gaine, S.P. & Rubin, L.J. Primary pulmonary hypertension. *Lancet* **352**, 719-725 (1998).
3. Rich, S. *et al.* Primary pulmonary hypertension: a national prospective study. *Ann. Intern. Med.* **107**, 216-223 (1987).
- 10 4. Loyd, J.E., Primm, R.K. & Newman, J.H. Familial primary pulmonary hypertension: clinical patterns. *Am. Rev. Respir. Dis.* **129**, 194-197 (1984).
5. Nichols, W.C. *et al.* Localisation of the gene for familial primary pulmonary hypertension to chromosome 2q31-32. *Nature Genet.* **15**, 277-280 (1997).
- Morse, J.H. *et al.* Mapping of familial pulmonary hypertension locus (*PPH1*) to  
15 chromosome 2q31-32. *Circulation* **95**, 2603-2606 (1997).
7. Massagué, J. & Chen, Y-G. Controlling TGF- $\beta$  signalling. *Genes Dev.* **14**, 627-644 (2000).
8. Wrana, J.L. *et al.* Two distinct transmembrane serine/threonine kinases from *Drosophila melanogaster* form an activin receptor complex. *Mol. Cell. Biol.* **14**, 944-  
20 950 (1994).
9. Carcamo, J., Zentella, A. & Massagué, J. Disruption of transforming growth factor beta signalling by a mutation that prevents transphosphorylation within the receptor complex. *Mol. Cell. Biol.* **15**, 1573-1581 (1995).
10. Gilboa, L. *et al.* Bone morphogenetic protein receptor complexes on the surface  
25 of live cells: A new oligomerization mode for serine/ threonine kinase receptors. *Mol. Biol. Cell.* **11**, 1023-1035 (2000).
11. Hadano, S. *et al.* A yeast artificial chromosome-based physical map of the juvenile amyotrophic lateral sclerosis (ALS2) critical region on human chromosome 2q33-q34. *Genomics* **55**, 106-112 (1999).

13. Kawabata, M., Chytil, A. & Moses, H.L. Cloning of a novel type II serine/threonine kinase receptor through interaction with the type I transforming growth factor-beta receptor. *J. Biol. Chem.* **270**, 5625-5630 (1995).
14. Liu, F., Ventura, F., Doody, J. & Massagué, J. Human type II receptor for bone morphogenic proteins (BMPs): extension of the two-kinase receptor model to the BMPs. *Mol. Cell. Biol.* **15**, 3479-3486 (1995).
15. Rosenzweig, B.L. *et al.* Cloning and characterization of a human type II receptor for bone morphogenetic proteins. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 7632-7636 (1995).
16. Botney, M.D., Bahadori, L. & Gold, L.I. Vascular remodeling in primary pulmonary hypertension. Potential role for transforming growth factor-beta. *Am. J. Pathol.* **144**, 286-295 (1994).
17. Marchuk, D.A. Genetic abnormalities in hereditary hemorrhagic telangiectasia. *Curr. Opin. Hematol.* **5**, 332-338 (1998).
18. Beppu, H., Minowa, O., Miyazono, K. & Kawabata, M. cDNA cloning and genomic organization of the mouse BMP type II receptor. *Biochem. Biophys. Res. Commun.* **235**, 499-504 (1997).
19. Wilkie, A.O. The molecular basis of genetic dominance. *J. Med. Genet.* **31**, 89-98 (1994).
21. Lu, S.L. *et al.* HNPCC associated with germline mutation in the TGF-beta type II receptor gene. *Nature Genet.* **19**, 17-18 (1998).
22. Lee, S.D. *et al.* Monoclonal endothelial cell proliferation is present in primary but not secondary pulmonary hypertension. *J. Clin. Invest.* **101**, 927-934 (1998).
- Shackleton, S. *et al.* LMNA, encoding lamin A/C, is mutated in partial lipodystrophy. *Nature Genet.* **24**, 153-156 (2000).

## Example 2

### Materials and Methods

#### Patients

- Patients (age range 14-55 years) were recruited through physicians at specialist pulmonary vascular clinics in the UK (n=35), France (n=13) and the USA (n=2). PPH

was defined by standard clinical methods, including cardiac catheterization revealing pulmonary hypertension (mean pulmonary artery pressure > 25 mm Hg) and a normal pulmonary artery wedge pressure, without other abnormalities such as lung disease, heart disease, pulmonary embolism or systemic disease such as connective tissue diseases. All studies were performed with consent and approval by the Leicestershire Health Authority Ethics Committee (England).

### Typical Presentation

At age 36, patient 10 a previously fit and well nulliparous white female, developed shortness of breath and reduced exercise tolerance. She had no previous history of tobacco consumption, nor use of appetite suppressants. A five generation detailed pedigree revealed no preceding family history of PPH. The following year, she was investigated for worsening breathlessness. On clinical examination, external appearance was normal, cardiac auscultation revealed a loud second pulmonary heart sound. A radiograph of the chest showed enlarged pulmonary arteries with 'pruning' of peripheral vessels, electrocardiogram had evidence of right ventricular strain and an echocardiogram revealed an enlarged right ventricle and moderate tricuspid regurgitation. An autoantibody screen and ventilation/ perfusion scan were normal. At right heart catheterisation, pulmonary artery pressure was 97/41 mmHg with a pulmonary wedge pressure of 8 mmHg, and a diagnosis of primary pulmonary hypertension was made. The patient was anti-coagulated on warfarin and commenced on a calcium channel blocking agent, diltiazem. Her condition deteriorated over the following year with increasing episodes of retro-sternal chest pain, haemoptysis, syncope and the development of peripheral oedema. Domiciliary oxygen was provided and she was assessed for heart-lung transplantation, which she received at age 41. Histology of the explanted lungs showed marked intimal expansion and atheromatous plaques in the main branches of pulmonary arteries. Distal arteries showed hypertrophied muscularised media, intimal expansion and obliteration, with formation of plexiform lesions. The alveoli and bronchi appeared normal. A year following transplant she returned to work and remains under follow-up on a regime of immunosuppression.

### DNA Sequence Analysis of BMPR2 gene

We obtained 10 to 20 ml of peripheral blood from each family member studied. DNA was isolated from whole blood as described elsewhere. Parental relationships were confirmed through the segregation analysis of 10 independent highly polymorphic markers. Protein coding sequences from exons 1 to 13 were amplified from genomic DNA using primers derived from intron sequence as described in Example 1. Genomic fragments amplified by the polymerase chain reaction (PCR) were sequenced with a dye-terminator cycle-sequence system (ABI 3700, Perkin-Elmer Applied Biosystems, Foster City, CA).

### Confirmation of Genotypes and Detection of Spontaneous (*de novo*) Mutations

Variants of the BMPR2 gene were identified by sequence analysis and, when possible, were independently confirmed by restriction endonuclease digestion. Relevant exons were PCR amplified using primers as described, digested with restriction enzymes (*Hae* III, *Taq* I, *Mse* I, *Fnu4H* I, New England Biolabs) according to the manufacturer's instructions, and size-separated on a 4% composite agarose gel (FMC BioProducts, Gibco BRL). The presence or absence of the sequence variants from available family members and at least 150 normal control chromosomes was determined by analysis of the restriction digest or direct sequencing results.

### Results

#### Analysis of the BMPR2 Gene

Sequencing of genomic DNA of the panel of sporadic PPH subjects demonstrated a variety of novel heterozygous mutations of the BMPR2 gene (Table 4). In patients 1 and 2, the nucleotide sequence revealed substitutions of guanine for adenine in exons 2 and 3 respectively. These change the sequences of codon 60 from TGC to TAC (patient 1) and codon 117 from TGT to TAT (patient 2); both changing a highly conserved encoded amino acid from cysteine to tyrosine (Table 4). As these mutations do not result in either gain or loss of a restriction site, genomic sequencing of parental samples demonstrated the presence of the mutation in the fathers of both sporadic patients.



In patient 8, genomic sequencing identified a substitution of thymine for cytosine in exon 11, changing the sequence of codon 483 from TGT to CGT and the encoded amino acid from cysteine to arginine (Table 2). No additional family members were available for study. In patients 6 and 10, deletion of an adenine in exon 9 and a  
5 guanine in exon 12 both lead to a change in the coding reading frame, and predict premature truncation of the 1038 amino-acid protein at codon positions 423 and 803 respectively. Analysis of samples from unaffected parents, either by direct sequencing of genomic DNA (patient 6) or restriction enzyme analysis with *Fnu4HI* (patient 10), demonstrated the absence of the mutation, confirming these patients had spontaneous  
10 mutations of the *BMPR2* gene (Table 4).

The possibility of incorrect paternity was excluded by the analysis of informative markers. The mutation observed in patient 10, was also detected in two further sporadic patients, 11 and 12 ascertained independently. No parental samples were available and the possibility of the patients having inherited the mutation from a  
15 common ancestor was excluded through the examination of genotypes from microsatellite markers from within and surrounding the *BMPR2* gene on chromosome 2.

In three additional patients, insertions of residues in the genomic sequence occurred. In patient 4, an additional thymine was detected in exon 6 at position 787  
20 (Table 4). In patient 7 both a guanine and adenine were inserted at nucleotide position 1247-8 of exon 9, while in patient 9 an adenine is inserted in exon 12 at position 1969 and confirmed through restriction digest analysis with *Mse* I (Table 4). Each of the mutations predicts premature truncation of the *BMPR-II* protein through shifts of the reading frame (Table 4). Parental material was not available for analysis for these  
25 subjects.

In patients 3 and 5, the substitution of cytosine for thymine occurred in exons 6 and 8 respectively (Table 4). In both patients the mutations result in the change of the encoded amino-acid arginine CGA to the stop signal TGA. These sequence changes were confirmed by restriction digest analysis of genomic DNA with *Hae*III and *Taq*I  
30 respectively; however samples from other family members were not available.

None of these sequence changes were detected in the analysis of a large panel of chromosomes from unrelated normal individuals, indicating that these mutations are not polymorphisms. Mutations of the entire coding sequence of the BMPR2 gene were also excluded in the remaining 38 patients diagnosed with sporadic PPH.

5           **Pulmonary artery myocytes from patients with familial and sporadic PPH exhibit abnormal responses to TGF- $\beta$  family ligands**

PPH myocytes exhibited specifically heightened  $^3\text{H}$ -thymidine incorporation to BMP2, a known BMPR-II ligand, and TGF- $\beta$ . In contrast, no increased DNA synthesis was observed in response to these peptides in cells from control subjects or patients  
10 with secondary pulmonary hypertension. Indeed, TGF- $\beta$  suppressed basal  $^3\text{H}$ -thymidine incorporation in pulmonary artery myocytes from controls. PDGF- $\beta$  stimulated  $^3\text{H}$ -thymidine incorporation by an equal amount in all cells, with no significant difference between patient groups. Growth arrested pulmonary artery myocytes from patients with PPH were used. Incubations were for 48 hours with  
15 [methyl]- $^3\text{H}$ -thymidine added for final 24 hours. All subjects were age-matched and patients had a comparable degree of pulmonary hypertension: PPH (mean pulmonary artery pressure 604 mmHg); SPH (655 mmHg). \* $p < 0.05$ , \*\* $p < 0.01$  compared with corresponding 0.1% FBS.

**References**

- 20           1. Rubin LJ. Primary Pulmonary Hypertension. The New England Journal of Medicine 1997; 336:111-117.
2. Gaine SP, Rubin LJ. Primary pulmonary hypertension. Lancet 1998; 352:719-725.
3. Rich S, Dantzker DR, Ayres SM, Bergofsky EH, Brundage BH, Detre  
25 KM, et al. Primary pulmonary hypertension. A national prospective study. Ann.Intern.Med. 1987; 107:216-223.
4. Loyd JE, Primm RK, Newman JH. Familial primary pulmonary hypertension: clinical patterns. Am.Rev.Respir.Dis. 1984; 129:194-197.
5. Machado RD, Pauciulo MW, Fretwell N, Veal C, Thomson JR, Güell  
30 CV, et al. A physical and transcript map based upon refinement of the critical interval

- for PPH1, a gene for familial primary pulmonary hypertension. *Genomics* 2000; In press.
6. Nichols WC, Koller DL, Slovis B, Foroud TM, Terry VH, Arnold ND, et al. Localization of the gene for familial primary pulmonary hypertension to chromosome 2q31-32. *Nat.Genet.* 1997; 15:277-280.
7. Morse JH, Jones AC, Barst RJ, Hodge SE, Wilhelmsen KC, Nygaard TG. Mapping of familial primary pulmonary hypertension locus (PPH1) to chromosome 2q31-q32. *Circulation* 1997; 95:2603-2606.
8. Deng Z, Haghighi F, Helleby L, Vanterpool K, Horn EM, Barst RJ, et al. Fine mapping of PPH1, a gene for familial primary pulmonary hypertension, to a 3 cM region on chromosome 2q33. *American Journal of Respiratory & Critical Care Medicine* 2000; 161:1055-1059.
9. The International PPH Consortium, Lane KB, Machado RD, Pauculo MW, Thomson JR, Phillips III JA, et al. Heterozygous germline mutations in a TGF- $\beta$  receptor, *BMPR2*, are the cause of familial primary pulmonary hypertension. *Nat.Genet.* 2000; In press.
10. Massague J, Chen YG. Controlling TGF- $\beta$  signaling. *Genes & Development* 2000; 14:627-644.
11. Loyd JE, Atkinson JB, Pietra GG, Virmani R, Newman JH. Heterogeneity of Pathologic Lesions in Familial Primary Pulmonary Hypertension. *Am.Rev.Respir.Dis.* 1988; 138:952-957.
12. Pietra GG, Edwards WD, Kay JM, Rich S, Kernis J, Schloo B, et al. Histopathology of primary pulmonary hypertension. A qualitative and quantitative study of pulmonary blood vessels from 58 patients in the National Heart, Lung, and Blood Institute, Primary Pulmonary Hypertension Registry. *Circulation* 1989; 80:1198-1206.
13. Elliott G, Alexander G, Leppert M, Yeates S, Kerber R. Coancestry in apparently sporadic primary pulmonary hypertension. *Chest* 1995; 108:973-977.
14. Morrell NW, Upton PD, Kotecha S, Huntley A, Yacoub MH, Polak JM, et al. Angiotensin II activates MAPK and stimulates growth of human pulmonary

artery smooth muscle via AT1 receptors. *American Journal of Physiology* 1999; 277:L440-L448

15. Rich S. Primary pulmonary hypertension. Executive summary from the world symposium. *Primary Pulmonary Hypertension*. World Health Organisation Publications 1998;
16. Marchuk DA. Genetic abnormalities in hereditary hemorrhagic telangiectasia. *Current Opinion in Hematology* 1998; 5:332-338.
17. Blobe GC, Schiemann WP, Lodish HF. Role of transforming growth factor b in human disease. *N Engl J Med* 2000; 342:1350-1358.
- 10 18. Mesa RA, Edell ES, Dunn WF, Edwards WD. Human immunodeficiency virus infection and pulmonary hypertension: two new cases and a review of 86 reported cases. *Mayo Clinic Proceedings* 1998; 73:37-45.
19. Abenhaim L, Moride Y, Brenot F, Rich S, Benichou J, Kurz X, et al. Appetite-suppressant drugs and the risk of primary pulmonary hypertension.
- 15 International Primary Pulmonary Hypertension Study Group [see comments]. *N.Engl.J.Med.* 1996; 335:609-616.
20. Gomez-Sanchez MA, Saenz dIC, Gomez-Pajuelo C, Martinez-Tello FJ, Mestre dJM, James TN. Clinical and pathologic manifestations of pulmonary vascular disease in the toxic oil syndrome. *Journal of the American College of Cardiology* 1991; 18:1539-1545.
21. Lee SD, Shroyer KR, Markham NE, Cool CD, Voelkel NF, Tudor RM. Monoclonal endothelial cell proliferation is present in primary but not secondary pulmonary hypertension. *Journal of Clinical Investigation* 1998; 101:927-934.
22. Lu SL, Kawabata M, Imamura T, Akiyama Y, Nomizu T, Miyazono K, et al. HNPCC associated with germline mutation in the TGF- $\beta$  type II receptor gene. *Nat.Genet.* 1998; 19:17-18.
- 25

TABLE 1

Family/Pt	Location	Mutation	Nucleotide change	Consequence	Segregation
+UK13	Exon 3	Deletion	355delA GCTGTTGTA	frameshift	<i>Bsp1286I</i>
UK09	Exon 3	C123R	367 TC	missense	<i>MseI</i>
UK21	Exon 4	Insertion	504insT GTTGCCTTT	frameshift	
FRA	Exon 6	Deletion	689/90delAA TGCTGTAAA	frameshift	DS
+UK06	Exon 8	C347T	G1042A	missense	DS
GER01	Exon 9	C420R	T1258C	missense	<i>AfaI/Bsp1407I/BsrGI/SspBI/MaeI</i>
+NL01	Exon 11	D485G	A1454G	missense	<i>AvaII</i>
GRE01	Exon 11	L512T	A1535 C	missense	
SWE01	Exon 12	R584X	C1749T	nonsense	
UK22	Exon 12	Insertion	2292insA ACCAAAAAA	frameshift	DS
UK11	Exon 12	Deletion	2579-2580delT ATTAATT	frameshift (PTC + 10 aa)	<i>AseI</i>
3' UK04	Exon 7	Deletion	958+3 delT AGGAGGTA	inactivates exon 7 donor splice site	
+US35	Exon 2	S73X	C218G	nonsense	DS
+US14	Exon 3	C118W	T354G	missense	<i>Bsp1286I</i>
+US33	Exon 12	R899X	C2695T	nonsense	<i>HaeIII</i>
+US55	Exon 12	Deletion	2579-2580delT ATTAATT	frameshift (PTC + 10 aa)	<i>AseI</i>
US94	Exon8	Deletion	1076delC GGCTGAC	frameshift (PTC+ 15 aa)	
US89	Exon 9	Deletion	1191/1192delTG GGACTGTG	frameshift (PTC + 48 aa)	
US80	Exon 9	Splice Defect	1129-3CG	inactivates exon 9 acceptor splice site	
US37	Exon 4	R147X	C428T	nonsense	
US49	Exon 11	N519K	T1557A	missense	DS
US79	Exon 12	Insertion	2408insTG TGGTGTG	frameshift (PTC + 3 aa)	
NOR01	Exon 3	C123S	T367A	missense	<i>MseI</i>
US50	Exon 8	R332X	C993T	nonsense	

TABLE 2

Patient	Location	Mutation	Nucleotide change	Consequence	Segregation	Mat	Pat	Centre
5226	Exon 2	C60Y	G179A	missense	DS	WT	C196Y	Manchester
3576	Exon 6	Insertion	TTTATAGTTT	frameshift	DS	Declined	Declined	Newcastle
5949	Exon 6	R211X	C631T	nonsense	<i>Hae</i> III	Alive, sample being arranged	Alive, sample being arranged	France
5591	Exon 8	R332X	C994T	nonsense	<i>Taq</i> I	Alive, to arrange sample	Alive, to arrange sample	CXH
5508	Exon 9	Insertion	GGGAGAGA 1247/48insGA	frameshift	DS	Alive, to arrange sample	?	Glasgow
5927	Exon 11	C483R	T1447C	missense	DS	Blood sample taken	?	France
5545	Exon 12	Insertion	TCTGCTTAA 1969insA	frameshift	<i>Mse</i> I	WT	RIP	Newcastle
5163	Exon 12	Deletion	CAATCAATG 2386delG	frameshift	<i>Fnu</i> 4HI	WT	WT	Harefield
5937	Exon 12	Deletion	CAATCAATG 2386delG	frameshift	<i>Fnu</i> 4HI	To request	To request	France
5943	Exon 12	Deletion	CAATCAATG 2386delG	frameshift	<i>Fnu</i> 4HI	To request	To request	France
Ut. 01	Exon 9	Deletion	GGGAGATA 1248delA	frameshift	DS	WT	WT	Utah
Ut. 11	Exon 3	C117Y	350G--A	missense	DS	WT	C117Y	Utah

Characteristics	1	2	3	4	5	6	7	8	9	10	11	12
Sex	F	M	F	F	F	M	M	F	F	F	F	M
Age (years)	23	31		27	35	29	42		34	43		
Age of onset (years)	17	25		22	29	22			30	36		
Family History	-	-	-	-	-	-	-	-	-	-	-	-
Therapy (vasodilators)	+	+	+	+	+	-	+	+	+	-	+	+
HLT	-	-	-	-	-	+	-	-	-	+	-	-
Mutation <i>BMPR2</i>	C60Y	C117Y	R211X	787insT	R332X	1248-delA	1247/48-insGA	C483R	1969-insA	2386-delG	2386-delG	2386-delG

Table 3. Clinical features of PPH.

Patient No.	Patient Identifier	Exon	Mutation Type	Nucleotide Change	Codon Position	Amino Acid Change	Inheritance	Restriction Enzyme
1	5226	2	Missense	G(179) A	60	Cys to Tyr	Paternal	-
2	Ut.11	3	Missense	G(350) A	117	Cys to Tyr	Paternal	
3	3576	6	Frameshift	787insT	263	PTC+3		
4	5949	6	Nonsense	C(631) T	211	Arg to Stop	-	<i>HaeIII</i>
5	5591	8	Nonsense	C(994) T	332	Arg to Stop	-	<i>TaqI</i>
6	Ut.01	9	Frameshift	1248delA	416	PTC+7	<i>de novo</i>	-
7	5508	9	Frameshift	1247/8ins GA	416	PTC+4	-	-
8	5927	11	Missense	T(1447) C	483	Cys to Arg	-	-
9	5545	12	Frameshift	1969insA	657	PTC+18	-	<i>MseI</i>
10	5163	12	Frameshift	2386delG	796	PTC+7	<i>de novo</i>	<i>Fnu4HI</i>
11	5937	12	Frameshift	2386delG	796	PTC+7	-	<i>Fnu4HI</i>
12	5943	12	Frameshift	2386delG	796	PTC+7	-	<i>Fnu4HI</i>
13	5597	1-6	Deletion					

Table 4. Mutations identified.



It must be noted that as used herein and in the appended claims, the singular forms "a ", "an", and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "a host cell" includes a plurality of such host cells, reference to "the antibody" is a reference to one or more antibodies and  
5 equivalents thereof known to those skilled in the art, and so forth.

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of skill in the art to which the disclosed invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention,  
10 the preferred methods, devices, and materials are as described.

## CLAIMS

We claim:

1. A method of identifying a subject having an increased susceptibility for developing pulmonary hypertension, comprising detecting a mutant Bone Morphogenic Protein Receptor II (BMPR-II) polypeptide or a mutated Bone Morphogenic Protein Receptor 2 (BMPR2) nucleic acid in the subject, thereby identifying a subject having an increased susceptibility for developing pulmonary hypertension.
2. The method of claim 1, wherein the subject having an increased susceptibility for developing pulmonary hypertension is identified by detecting a mutated BMPR2 nucleic acid in the subject.
3. The method of claim 2, wherein the mutated BMPR2 nucleic acid encodes a mutant BMPR-II polypeptide.
4. The method of claim 2 wherein the mutated BMPR2 nucleic acid comprises a missense mutation.
5. The method of claim 2, wherein the mutated BMPR2 nucleic acid comprises a nonsense mutation.
6. The method of claim 2, wherein the mutated BMPR2 nucleic acid comprises a deletion mutation.
7. The method of claim 2, wherein the mutated BMPR2 nucleic acid comprises an insertion mutation.
8. The method of claim 2, wherein the mutated BMPR2 nucleic acid comprises a truncation mutation.
9. The method of claim 8, wherein the mutated BMPR2 nucleic acid is truncated at a nucleotide position of the sequence set forth in SEQ ID NO:1 which is 3' to nucleotide position 2695.
10. The method of claim 2, wherein the mutated BMPR2 nucleic acid comprises a nucleotide sequence that differs from the sequence set forth in SEQ ID NO:1.
11. The method of claim 1, wherein the mutated BMPR2 nucleic acid or mutant BMPR-II polypeptide has a sequence associated with pulmonary hypertension.

12. The method of claim 11, wherein the mutated BMPR2 nucleic acid comprises a mutation at a nucleotide position of the sequence set forth in SEQ ID NO:1 selected from the group consisting of 218, 354, 355, 367, 428, 504, 689, 958, 993, 1042, 1076, 1129, 1191, 1258, 1454, 1535, 1557, 1749, 2292, 2408, 2579, and 2695.

13. The method of claim 12, wherein the mutation in the mutated BMPR2 nucleic acid results in a non-conservative substitution in the amino acid sequence encoded by the nucleic acid.

14. The method of claim 13, wherein the mutation in the mutated BMPR2 nucleic acid results in a non-conservative substitution at a Cys residue encoded by the nucleotide sequence set forth in SEQ ID NO:1.

15. The method of claim 13, wherein the mutation is selected from the group consisting of C 218 G, T 354 G, T 367 C, T 367 A, C 428 T, C 993 T, G 1042 A, T 1258 C, A 1454 G, A 1535 C, T 1557 A, C 2695 T.

16. The method of claim 1, wherein the subject having an increased susceptibility for developing pulmonary hypertension is identified by detecting a BMPR2 nucleic acid having a sequence associated with pulmonary hypertension.

17. The method of claim 16, wherein the BMPR2 nucleic acid having a sequence associated with pulmonary hypertension comprises a truncation mutation.

18. The method of claim 16, wherein the BMPR2 nucleic acid having a sequence associated with pulmonary hypertension comprises a missense mutation.

19. The method of claim 16 wherein the BMPR2 nucleic acid having a sequence associated with pulmonary hypertension comprises a nonsense mutation.

20. The method of claim 16, wherein the BMPR2 nucleic acid having a sequence associated with pulmonary hypertension comprises a deletion mutation.

21. The method of claim 16, wherein the BMPR2 nucleic acid having a sequence associated with pulmonary hypertension comprises a nucleic acid sequence having an insertion mutation.

22. The method of claim 16, wherein the BMPR2 nucleic acid having a sequence associated with pulmonary hypertension encodes a mutant BMPR-II polypeptide.

23. The method of claim 22, wherein the mutant BMPR-II polypeptide comprises at least one mutation at an amino acid position of the sequence set forth in SEQ ID NO:2.

24. The method of claim 22, wherein the BMPR-II polypeptide comprises at least one mutation at an amino acid position of the sequence set forth in SEQ ID NO:2 selected from the group consisting of 73, 118, 123, 143, 332, 348, 420, 485, 512, 519, and 899.

25. The method of claim 22, wherein the BMPR-II polypeptide comprises at least one mutation at an amino acid position of the sequence set forth in SEQ ID NO:2 selected from the group consisting of a Trp residue at amino acid position 118, an Arg residue at amino acid position 123, a Ser residue at amino acid position 123, a Leu residue at amino acid position 143, an Ile residue at amino acid position 348, an Arg residue at amino acid position 420, an Ala residue at amino acid position 485, a Gln residue at amino acid position Gln, and a Lys residue at amino acid position 519.

26. The method of claim 22, wherein the BMPR-II polypeptide having a sequence associated with pulmonary hypertension terminates prematurely.

27. The method of claim 26, wherein the BMPR-II polypeptide having a sequence associated with pulmonary hypertension terminates at an amino acid position of the sequence set forth in SEQ ID NO:2 which is N-terminal to amino acid position 73, 332, or 899.

28. The method of claim 22, wherein the BMPR-II polypeptide having a sequence associated with pulmonary hypertension has a non-conservative amino acid substitution of at least one amino acid residue of a BMPR-II having the amino acid sequence set forth in SEQ ID NO:2.

29. The method of claim 22, wherein the BMPR-II polypeptide having a sequence associated with pulmonary hypertension has a non-conservative amino acid substitution of at least one Cys residue of a BMPR-II having the amino acid sequence set forth in SEQ ID NO:2.

30. The method of claim 22, wherein the BMPR-II polypeptide having a sequence associated with pulmonary hypertension has a non-conservative amino acid

substitution of at least one Pro residue of a BMPR-II having the amino acid sequence set forth in SEQ ID NO:2.

31. The method of claim 22, wherein the BMPR-II polypeptide having a sequence associated with pulmonary hypertension has a non-conservative amino acid substitution of at least one Lys residue of a BMPR-II having the amino acid sequence set forth in SEQ ID NO:2.

32. The method of claim 22, wherein the BMPR-II polypeptide having a sequence associated with pulmonary hypertension has a non-conservative amino acid substitution of at least one Arg residue of a BMPR-II having the amino acid sequence set forth in SEQ ID NO:2.

33. The method of claim 22, wherein the BMPR-II polypeptide having a sequence associated with pulmonary hypertension has a non-conservative amino acid substitution of at least one Asp residue of a BMPR-II having the amino acid sequence set forth in SEQ ID NO:2.

34. The method of claim 22, wherein the BMPR-II polypeptide having a sequence associated with pulmonary hypertension has a non-conservative amino acid substitution of at least one Glu residue of a BMPR-II having the amino acid sequence set forth in SEQ ID NO:2.

35. The method of claim 1, wherein the subject having an increased susceptibility for developing pulmonary hypertension is identified by detecting a mutant BMPR-II polypeptide in the subject.

36. The method of claim 35, wherein the mutant BMPR-II polypeptide terminates prematurely.

37. The method of claim 36, wherein the mutant BMPR-II polypeptide terminates at an amino acid position of the sequence set forth in SEQ ID NO:2 which is N-terminal to amino acid position 73, 332, or 899.

38. The method of claim 35, wherein the mutant BMPR-II polypeptide comprises a non-conservative substitution at an amino acid position.

39. The method of claim 38, wherein the mutant BMPR-II polypeptide comprises a non-conservative substitution for a Cys residue.

40. The method of claim 38, wherein the non-conservative substitution is selected from the group consisting of a Trp residue at amino acid position 118, an Arg residue at amino acid position 123, a Ser residue at amino acid position 123, a Leu residue at amino acid position 143, an Ile residue at amino acid position 348, an Arg residue at amino acid position 420, an Ala residue at amino acid position 485, a Gln residue at amino acid position Gln, and a Lys residue at amino acid position 519.

41. The method of claim 35, wherein the mutant BMPR-II polypeptide comprises an amino acid sequence that differs from the sequence set forth in SEQ ID NO:2.

42. The method of claim 41, wherein the mutant BMPR-II polypeptide comprises a mutation at an amino acid position of the sequence set forth in SEQ ID NO:2 selected from the group consisting of 73, 118, 123, 143, 332, 348, 420, 485, 512, 519, and 899.

43. The method of claim 35, wherein the BMPR-II polypeptide comprises at least one mutation at an amino acid position of the sequence set forth in SEQ ID NO:2 selected from the group consisting of 73, 118, 123, 143, 332, 348, 420, 485, 512, 519, and 899.

44. The method of claim 35, wherein the BMPR-II polypeptide comprises at least one mutation at an amino acid position of the sequence set forth in SEQ ID NO:2 selected from the group consisting of a Trp residue at amino acid position 118, an Arg residue at amino acid position 123, a Ser residue at amino acid position 123, a Leu residue at amino acid position 143, an Ile residue at amino acid position 348, an Arg residue at amino acid position 420, an Ala residue at amino acid position 485, a Gln residue at amino acid position Gln, and a Lys residue at amino acid position 519.

45. The method of claim 1, wherein the subject having an increased susceptibility for developing pulmonary hypertension is identified by detecting a BMPR-II polypeptide having a sequence associated with pulmonary hypertension in the subject.

46. The method of claim 45, wherein the BMPR-II polypeptide having a sequence associated with pulmonary hypertension terminates prematurely.

47. The method of claim 46, wherein the BMPR-II polypeptide having a sequence associated with pulmonary hypertension terminates at an amino acid position of the sequence set forth in SEQ ID NO:2 which is N-terminal to amino acid position 73, 332, or 899.

48. The method of claim 45, wherein the BMPR-II polypeptide having a sequence associated with pulmonary hypertension has a non-conservative amino acid substitution of at least one amino acid residue of a BMPR-II having the amino acid sequence set forth in SEQ ID NO:2.

49. The method of claim 45, wherein the BMPR-II polypeptide having a sequence associated with pulmonary hypertension has a non-conservative amino acid substitution of at least one Cys residue of a BMPR-II encoded by the nucleotide sequence set forth in SEQ ID NO:1.

50. The method of claim 45, wherein the BMPR-II polypeptide having a sequence associated with pulmonary hypertension has a non-conservative amino acid substitution of at least one Pro residue of the wild-type BMPR-II encoded by the nucleotide sequence set forth in SEQ ID NO:1.

51. The method of claim 45, wherein the BMPR-II polypeptide having a sequence associated with pulmonary hypertension has a non-conservative amino acid substitution of at least one Lys residue of the wild-type BMPR-II encoded by the nucleotide sequence set forth in SEQ ID NO:1.

52. The method of claim 45, wherein the BMPR-II polypeptide having a sequence associated with pulmonary hypertension has a non-conservative amino acid substitution of at least one Arg residue of the wild-type BMPR-II encoded by the nucleotide sequence set forth in SEQ ID NO:1.

53. The method of claim 45, wherein the BMPR-II polypeptide having a sequence associated with pulmonary hypertension has a non-conservative amino acid substitution of at least one Asp residue of the wild-type BMPR-II encoded by the nucleotide sequence set forth in SEQ ID NO:1.

54. The method of claim 45, wherein the BMPR-II polypeptide having a sequence associated with pulmonary hypertension has a non-conservative amino acid

substitution of at least one Glu residue of the wild-type BMPR-II encoded by the nucleotide sequence set forth in SEQ ID NO:1.

55. The method of claim 1, wherein the mutant BMPR-II polypeptide or mutated BMPR2 nucleic acid is due to a familial mutation.

56. The method of claim 1, wherein the mutant BMPR-II polypeptide or mutated BMPR2 nucleic acid is due to a sporadic mutation.

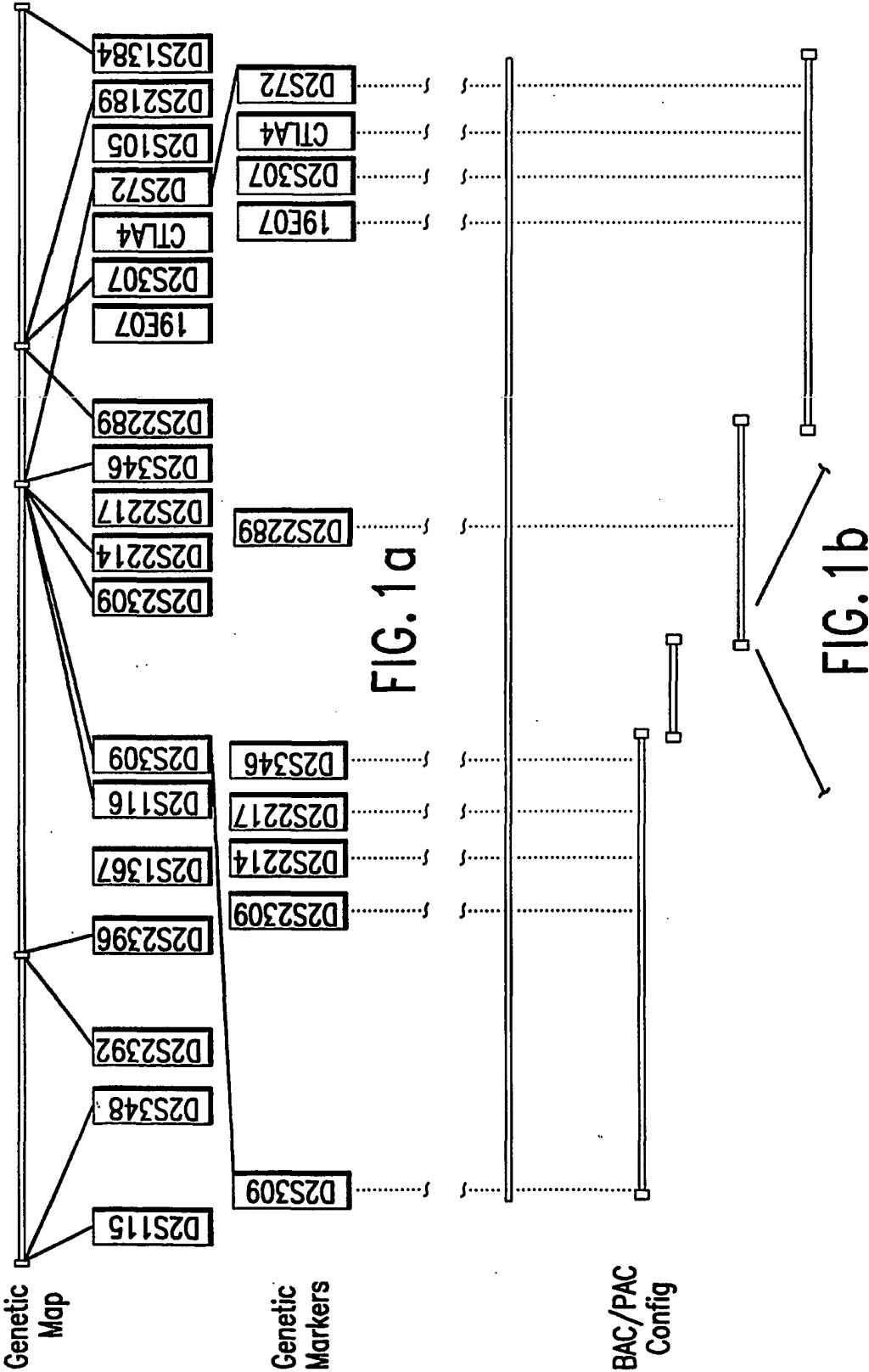
57. The method of claim 1, wherein the mutated BMPR2 nucleic acid has a sequence associated with pulmonary hypertension, wherein the mutated BMPR2 nucleic acid results in altered BMPR2 RNA function.

58. The method of claim 57, wherein the altered BMPR2 RNA function is reduced BMPR2 mRNA production, altered processing of BMPR2 RNA, or increased BMPR2 RNA instability.

59. The method of claim 58, wherein the altered BMPR2 RNA function is altered splicing of BMPR2 RNA.

60. A kit for identifying a subject having an increased susceptibility for developing pulmonary hypertension, comprising reagents for detecting a mutant Bone Morphogenic Protein Receptor II (BMPR-II) polypeptide or a mutated BMPR2 nucleic acid in the subject.





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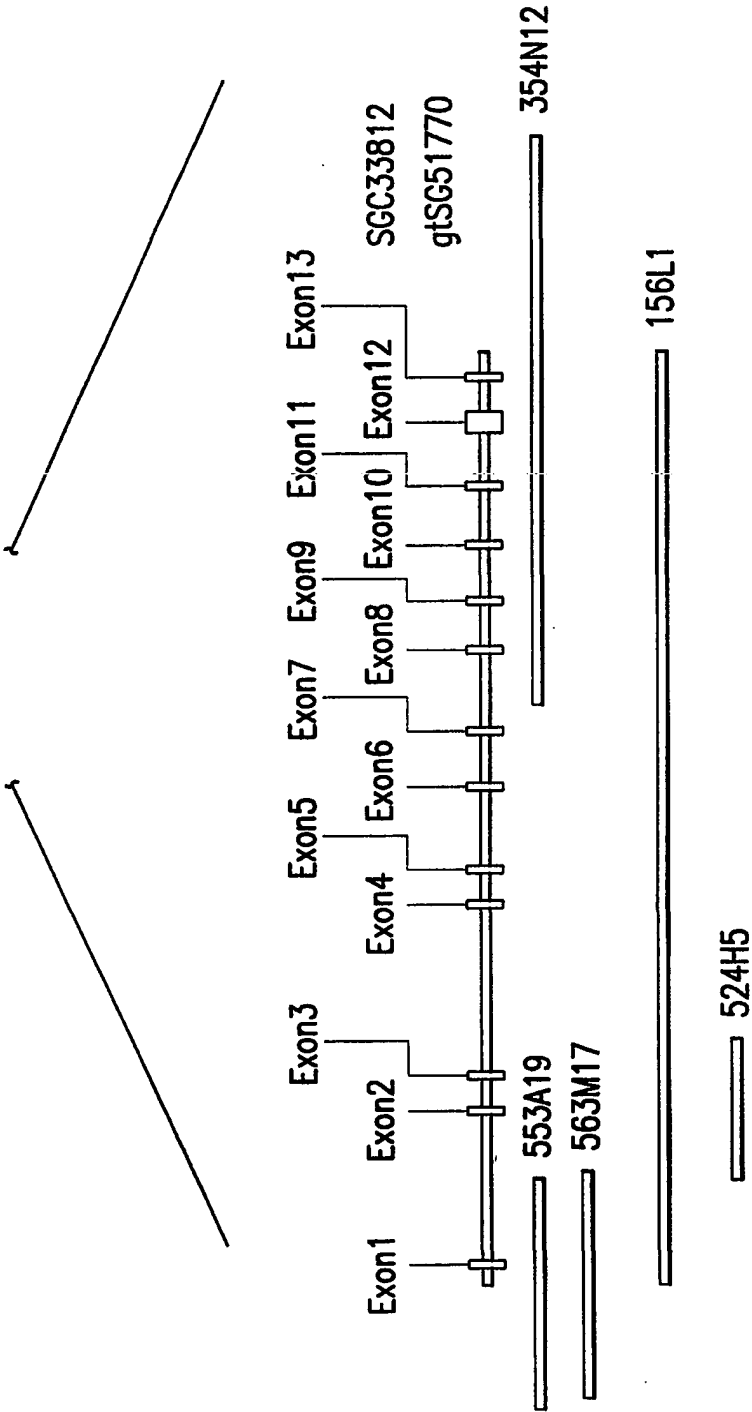


FIG. 1c

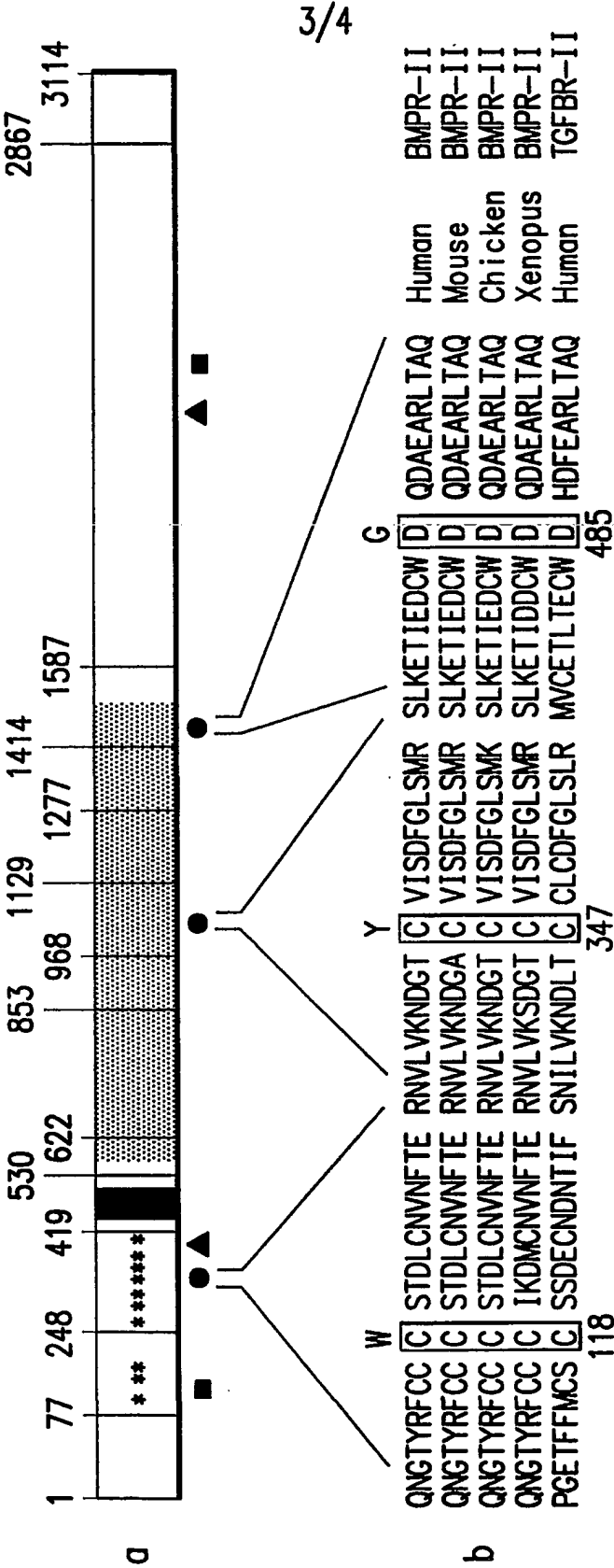


FIG.2

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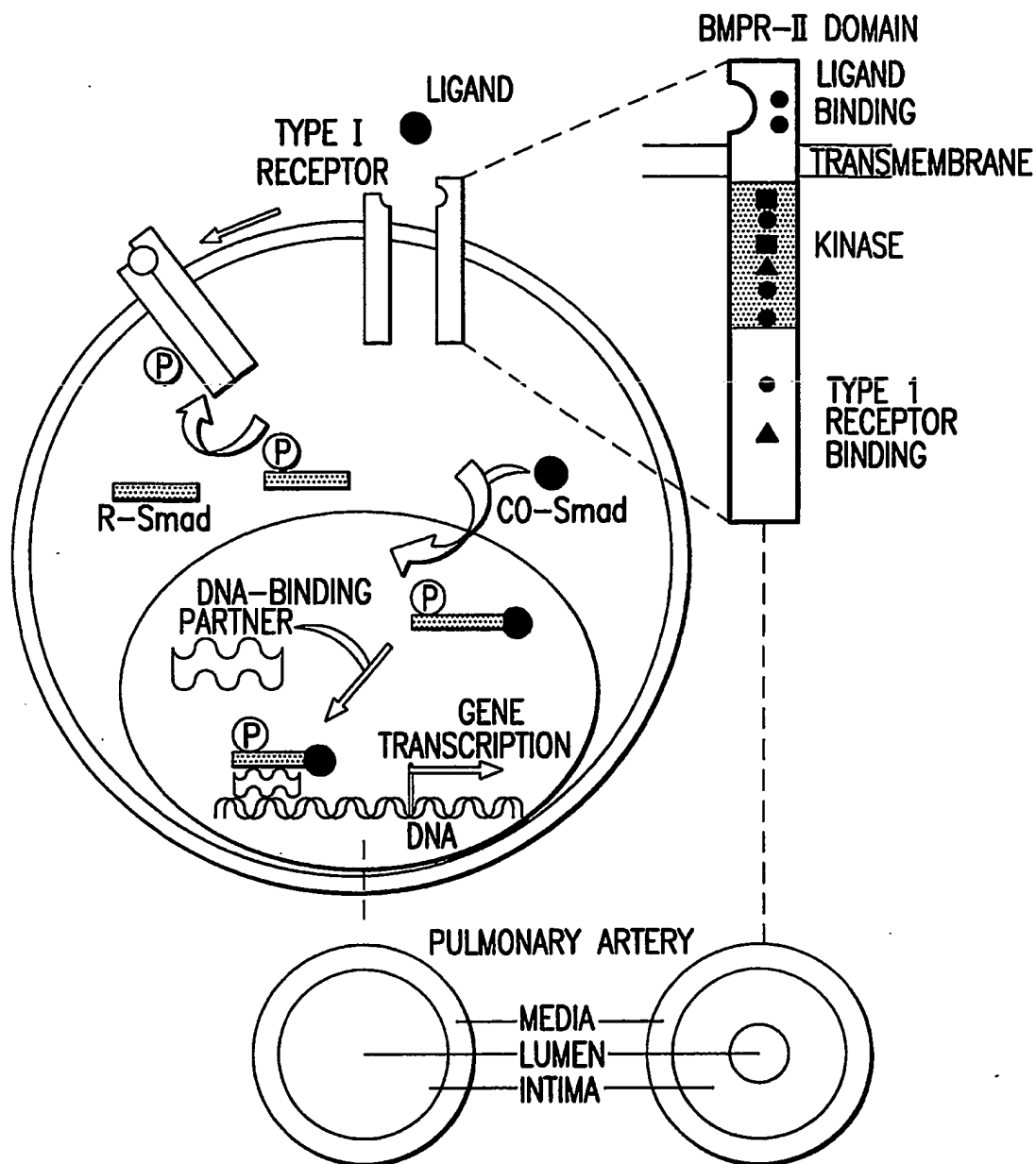


FIG.3

## SEQUENCE LISTING

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 Children's Hospital Medical Center  
 Advanced Research & Technology Institute  
 University of Leicester

<120> METHOD OF DIAGNOSING PULMONARY  
 HYPERTENSION

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<150> 60/220,133

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acc atc ctg ctg gtc agc act gcg gct gct tgc cag aat caa gaa cgg	96
Thr Ile Leu Leu Val Ser Thr Ala Ala Ala Ser Gln Asn Gln Glu Arg	
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cta tgt gcg ttt aaa gat ccg tat cag caa gac ctt ggg ata ggt gag	144
Leu Cys Ala Phe Lys Asp Pro Tyr Gln Gln Asp Leu Gly Ile Gly Glu	
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Lys Gln Gly Cys Trp Ser His Ile Gly Asp Pro Gln Glu Cys His Tyr	
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Pro	Ser	Lys	Pro	Arg	Arg	Ala	Gln	Arg	Pro	Asn	Ser	Leu	Asp	Leu	Ser
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Ala	Thr	Asn	Val	Leu	Asp	Gly	Ser	Ser	Ile	Gln	Ile	Gly	Glu	Ser	Thr
945					950					955					960

Gln Asp Gly Lys Ser Gly Ser Gly Glu Lys Ile Lys Lys Arg Val Lys  
                                   965                                  970                                  975  
 Thr Pro Tyr Ser Leu Lys Arg Trp Arg Pro Ser Thr Trp Val Ile Ser  
                                   980                                  985                                  990  
 Thr Glu Ser Leu Asp Cys Glu Val Asn Asn Asn Gly Ser Asn Arg Ala  
                                   995                                  1000                                  1005  
 Val His Ser Lys Ser Ser Thr Ala Val Tyr Leu Ala Glu Gly Gly Thr  
                                   1010                                  1015                                  1020  
 Ala Thr Thr Met Val Ser Lys Asp Ile Gly Met Asn Cys Leu  
 1025                                  1030                                  1035

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<220>  
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 agctaggtcc tctcatcagc 20

<210> 4  
 <211> 20  
 <212> DNA  
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<400> 4  
 cagccgcagt gctgaccagc 20

<210> 5  
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<400> 5  
 gtcattcgga taagacaaag 20

<210> 6  
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 tttaacatac tcccatgtcc 20

<210> 7  
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<400> 7  
tagcttacac gtactctcac 20

<210> 8  
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<400> 8  
cctggcttca accttgaatg 20

<210> 9  
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gggtacagcc tttctaaagg 20

<210> 10  
<211> 20  
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<400> 10  
gatactattg aggctgggtg 20

<210> 11  
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<400> 11  
gctgctaatac tttctgcagc 20

<210> 12  
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<400> 12  
gaatgaagtc actgttccag 20

<210> 13  
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Synthetic Construct

<400> 13  
cagagagctg tagcattctg 20

<210> 14  
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<400> 14  
aagtgatcca cctgccttag 20

<210> 15  
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<400> 15  
actcttcatg ttaaagtgag 20

<210> 16  
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Synthetic Construct

<400> 16  
ctttgaagat ataattaaaa tttcc 25

<210> 17  
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Synthetic Construct

<400> 17  
cacctggcca gtagatgttt 20

<210> 18  
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<400> 18  
tgttcaatag tcccttttat tcattg 26

<210> 19  
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<400> 20  
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<210> 21  
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<400> 21  
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<210> 22  
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Synthetic Construct

<400> 22  
gcctgaaggg gatgaaaaa 19

<210> 23  
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Synthetic Construct

<400> 23  
ccacaccct tagggtctta 20

<210> 24  
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<212> DNA  
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Synthetic Construct

<400> 24  
cacatggttt gacatgtact ttg 23

<210> 25  
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<220>  
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Synthetic Construct

<400> 25  
catcagagct ttccttgagg tt 22

<210> 26  
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<212> DNA  
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<220>  
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Synthetic Construct

<400> 26  
cagagtggtt aaatttgagg 20



<210> 27  
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Synthetic Construct

<400> 27  
tctacctgcc acaccattca 20

<210> 28  
<211> 21  
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Synthetic Construct

<400> 28  
tggaacacaa caagctagac c 21

<210> 29  
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Synthetic Construct

<400> 29  
ccccaaaaga cacacaggag 20

<210> 30  
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Synthetic Construct

<400> 30  
tgaatggtgt ggcaggtaga 20

<210> 31  
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<212> DNA  
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Synthetic Construct

<400> 31  
gctgacagga ggataaagca 20

<210> 32  
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<212> DNA  
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Synthetic Construct

<400> 32  
caccctcctg agacattggt 20

<210> 33  
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<212> DNA  
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Synthetic Construct

<400> 33  
acccaatatg ccaatgggac 20

<210> 34  
<211> 20  
<212> DNA  
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Synthetic Construct

<400> 34  
ttcgccacct tctagtggct 20

<210> 35  
<211> 23  
<212> DNA  
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Synthetic Construct

<400> 35  
catgtggtaa actgaaaagc tca 23

<210> 36  
<211> 23  
<212> DNA  
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<220>  
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Synthetic Construct

<400> 36  
ttgagaccac tttgatacac aca 23